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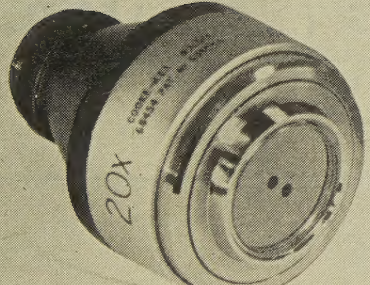

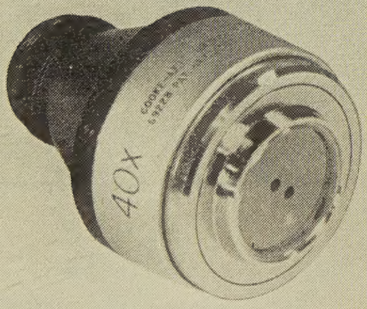

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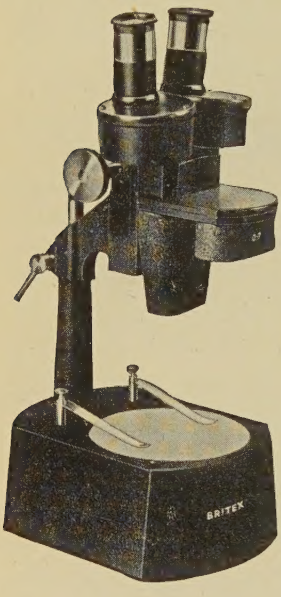

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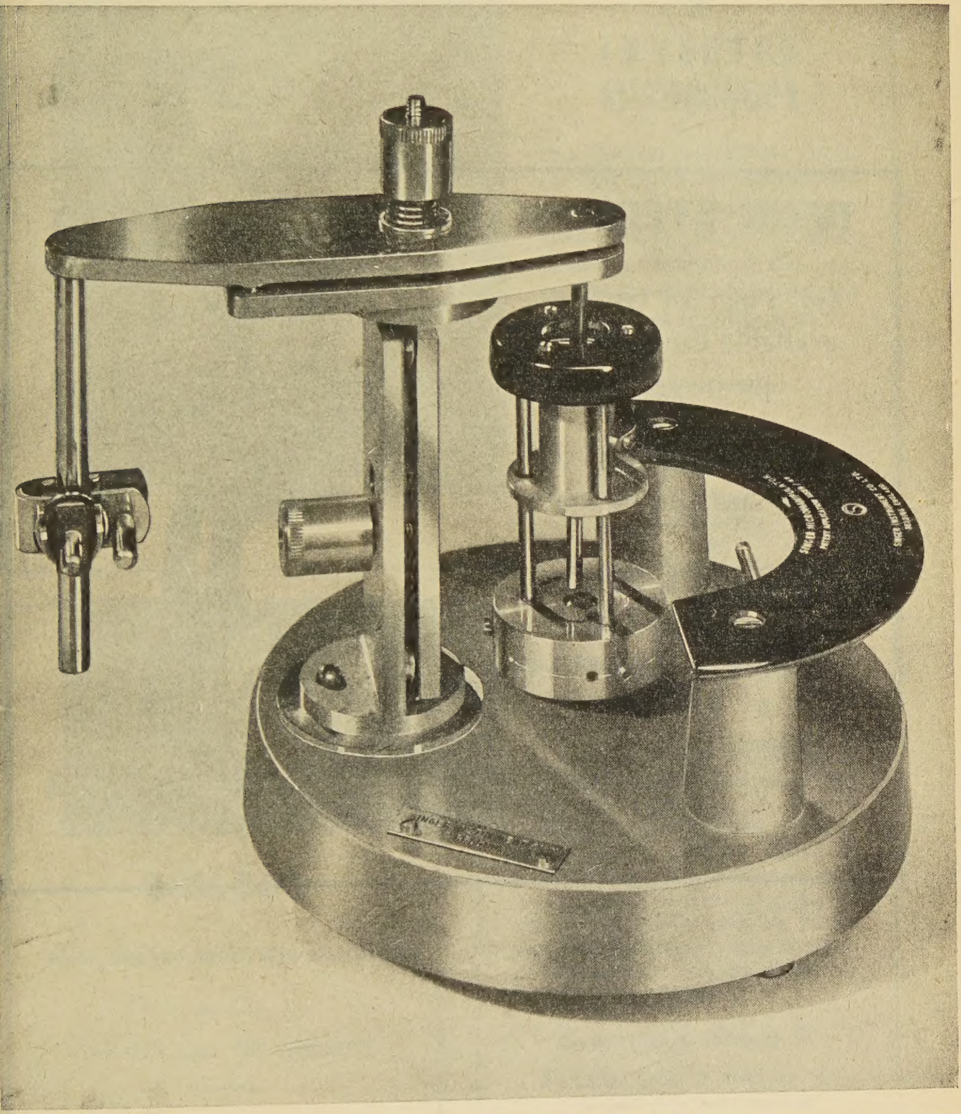

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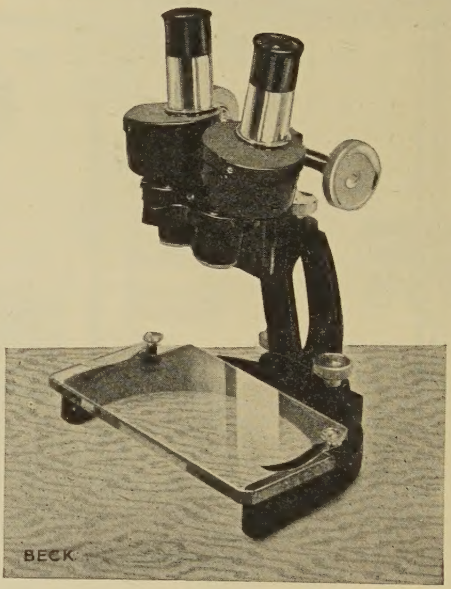

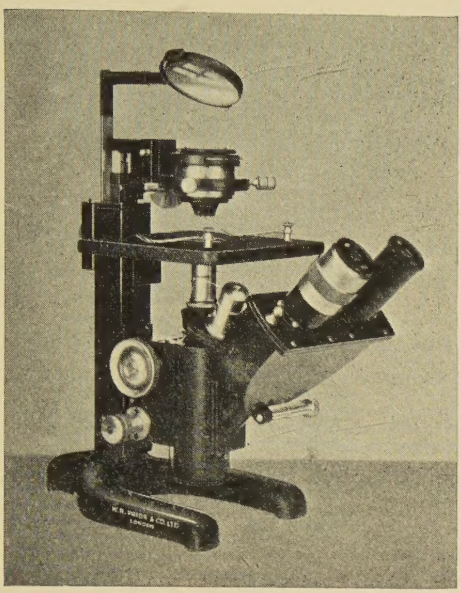

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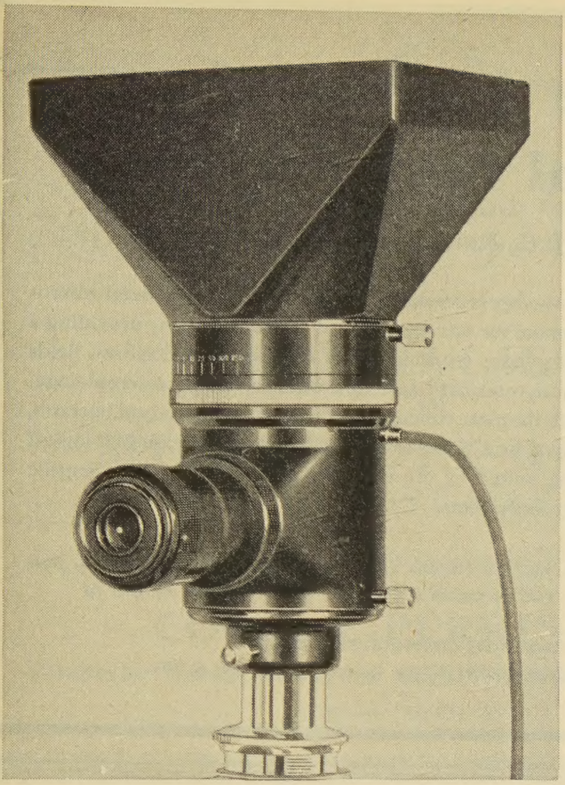

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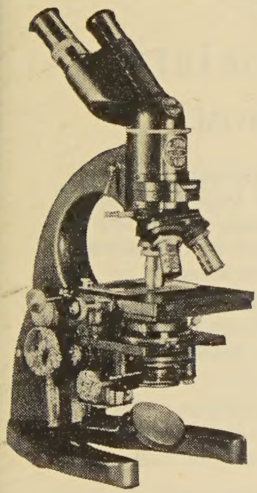

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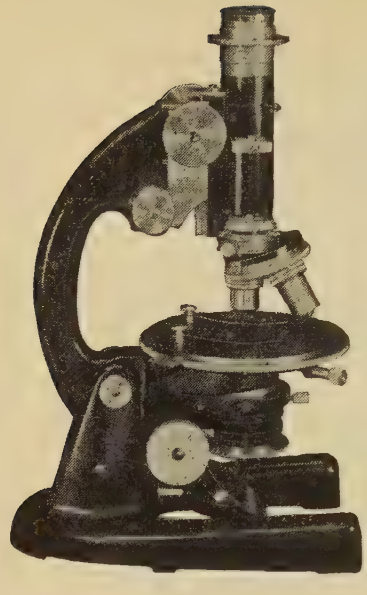

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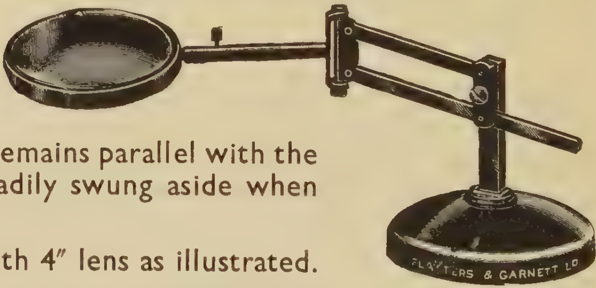

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issues for Assessing Histochemical Methods for Calcium

By S. M. McGEE-RUSSELL

rom the Department of Zoology and Comparative Anatomy, University Museum, Oxford.
Present address: Department of Zoology, Birkbeck College, Malet Street, London, W.C. 1)

With one plate (fig. 1)

SUMMARY

An account is given of three tissues which are suitable for use as standard objects hen attempting methods that are said to reveal calcium salts in tissues. The tissues e the digestive gland of the snail *Helix*, the gut diverticulum of the crab *Carcinus*, d the left colleterial gland of the cockroach *Periplaneta*. They contain respectively lcium carbonate, calcium phosphate, and calcium oxalate deposits. The deposits e little, if at all, contaminated with other calcium salts.

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INTRODUCTION

T is desirable that histochemical methods be assessed critically. Histochemical methods should be reproducible in a test-tube and follow known hemical criteria, and should produce consistent results in sections of animal r plant tissues. For test purposes such consistent results should be related to e known presence of the indicated substance in the tissue in reasonable uantity, as shown by chemical or biochemical analysis. If possible the distriution of the substance in the tissue should be indicated by criteria other han the results of the histochemical method alone, if that tissue is to be used s a true 'test' of the method. In the tissue the substance should be present in condition neither 'masked' nor 'bound', so that its reactions parallel the eactions which it gives as a single substance in a test-tube. These requireents are hard to meet for many of the substances for which histochemical ethods have been suggested. It is a peculiar difficulty of histochemical ethods that they have to be carried out on substances which are in close assoiation with a meshwork of precipitated proteins, and other substances. The

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spurious results which may result from this association are well known. However, it is because of this difficulty that critiques of methods should be carried out upon tissue sections as well as models, if possible.

The conditions outlined can be met for some substances, and this paper gives an account of three tissues from invertebrate animals which can be used satisfactorily as standard objects when trying out or criticizing methods said to reveal calcium salts in tissues. These tissues are preferable to the bone in young vertebrates, which has been used by some workers (e.g. Stock, 1949), as the matrix of bone has special staining properties which may give false differential colourings unrelated to the calcium present. The tissues may be used in conjunction with models of the type used by Cameron (1930) and give parity of results, and allow one to form a better idea of the precision of the cytological localization.

MATERIAL

The three tissues are the digestive gland of the snail *Helix* (either *Helix aspersa* or *H. pomatia*), the gut diverticulum of the crab *Carcinus maenas*, and the left colleterial gland of the cockroach *Periplaneta americana*. The snails were obtained in the neighbourhood of Oxford, and were kept in the laboratory on a diet of cabbage. The crabs were obtained fresh from Plymouth. The cockroachs were from a stock maintained by Dr. P. C. J. Brunet at Oxford.

The main requirement of the investigation of these three tissues as suitable test objects was to establish as clearly as possible the presence of calcium deposits in them and the nature of the calcium salts present.

THE IDENTIFICATION OF CALCIUM IN THE TEST TISSUES

Three alternative techniques were available to identify calcium in the tissues: microchemical methods applied to histological slides; microincineration of histological sections and application of microchemical tests to the ash; differential centrifugation of the homogenized tissue and application of chemical tests to the fractions, if microscopically identifiable as corresponding to elements in the tissue. All these methods were employed.

Lison (1936, 1953) states that the microchemical method of Schujeninoff is the only completely specific method for the detection of calcium. The section is treated with dilute sulphuric acid mixed with alcohol on the coverslip. In the presence of large quantities of calcium salts characteristic rosettes of gypsum crystals are formed. I found that this reaction does not occur strikingly with the three tissues in section, before incineration. However, after microincineration, the nature of the white ash is in the first instance a good indication of the metal present, and if dilute sulphuric acid is applied to the ash, the rapid formation of gypsum crystals is certain evidence of the presence of calcium.

In all three of the test tissues, microincineration produced white ash at the supposed sites of calcium. Application of dilute sulphuric acid resulted in the rapid formation of gypsum crystals, further confirming the presence of calcium. The localization of the ash is badly disturbed by the application of the acid, and there is a tendency for the gypsum crystals to develop at the edges of the acid drop rather than in any relation to the previous distribution of the ash, as noted by Manigault (1936–7). Nevertheless, the sites of calcium are sufficiently clearly indicated (fig. 1, B).

THE NATURE AND DISTRIBUTION OF THE CALCIUM SALTS

The digestive gland of Helix

The detailed histology of the digestive gland is complex. However, the most easily identifiable cell type is the calcium cell, *Kalkzelle*, or 'lime cell'. The last term is a misnomer, unfortunately used in the literature, for it is not likely that the animal isolates calcium oxide as such.

The calcium cells occur throughout the epithelium of the digestive tubules, in between the digestive cells. They are usually about 50 μ across at the base where the cells are attached to the basement membrane and connective tissue sheath. They are sometimes roughly triangular in outline in sections, being shorter than the adjacent digestive cells, which are usually about 100 μ in height and appear to squeeze the calcium cells slightly. Calcium cells in teased preparations of living tissue become spherical, with a diameter of about 50 to 60 μ, and are therefore about as wide as they are tall when in place in the epithelium. The large oval nucleus is central in the cell. The long axis of the nucleus is about 10 to 20 μ, and the nucleus possesses one, two, or three well-marked nucleoli and an abundance of karyosomes (fig. 1, A). The surrounding calcium spherules may give the nucleus an indented appearance, which may have led Cretin (1923) to describe it as resembling a chestnut. The calcium spherules pack the cytoplasm of the calcium cells. They are usually about 4 to 5 μ in diameter and are perfectly spherical. However, they may vary in size down to 1 μ or less. They are highly refringent and show in some cases internal structure in addition to the external protein coat which they all possess. The differentially centrifuged spherules may be decalcified with dilute acid under the coverslip, and the external protein coats remain spherical and undistorted, but are only visible by phase-contrast microscopy. In their characters the calcium spherules perfectly parallel the spherules figured by Waterhouse (1950), and their form is typical of the calcospherites which are found in a variety of invertebrates (Keilin, 1921).

Review of the literature quickly establishes that there are two schools of thought on the nature of the calcium salt composing the spherules in the calcium cells, as has recently been pointed out by Fretter (1952). Robertson (1941) in his extensive review of calcium in invertebrates identifies the spherules as composed of calcium phosphate, in accordance with such authors as Sioli (1935) and Krijgsman (1928). More recently Wagge (1948) and Heaysman (1951) found that the spherules were principally composed of calcium carbonate, and this view was previously held by Barfurth (1880) and others. The accumulation of radioactive phosphorus in the calcium cells has been established by Fretter (1952), and this author suggested that there was probably a balance between phosphate and carbonate in them. This amiable compromise resolves the controversy, and it is certainly a likely condition. However, it is difficult with the radioautographic technique used by Fretter to determine whether the phosphorus is accumulated actually within the spherules, or whether it is in the cytoplasm immediately surrounding them, which is another possibility.

The spherules showed the following properties:

(*a*) a weak birefringence between crossed polaroids, with the form of a black polarization cross, indicating a radial arrangement of micellae within the spherules;
(*b*) effervescence with dilute acids such as hydrochloric acid. The gas was identified as carbon dioxide;
(*c*) after fixation and embedding the spherules were very noticeable objects in sections, because of their refringency, and under a Watson 2 mm fluorite objective they appeared to have a violet-pink colour, probably as a consequence of their optical properties.

Tests with ammonium molybdate solution, and with silver nitrate, did not produce any significant results.

The weakness of the anisotropy of the spherules is surprising if the calcium is present as carbonate, unless it is in the form termed 'amorphous' (Prenant, 1927). However, the amount of effervescence is certainly sufficient to suggest the presence of a high quantity of carbonate. Heaysman (1951) goes so far as to identify the salt as aragonite upon the basis of Meigen's reaction. In a cold solution of cobalt nitrate calcite does not colour, and colours very little in a warm solution, whereas aragonite, especially with the hotter solution, is

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FIG. 1 (plate). In all the cases illustrated, the tissue was fixed in formaldehyde / alcohol (50: 50) and embedded in paraffin. Details of the histochemical methods used to obtain the results illustrated will be published later; see also McGee-Russell, 1954.

A, digestive gland of snail. Calcium deposits were coloured with sodium rhodizonate reagent, and the tissue was counterstained with toluidine blue. The photograph shows the typical appearance of a calcium cell.

B, digestive gland of snail. Deparaffinized section after microincineration, showing the effect of the addition of dilute sulphuric acid to the white ash produced on the site of calcium cells. Dark-ground illumination.

C, sub-mantle tissues, covering the digestive gland of the snail. Calcium deposits coloured with sodium rhodizonate reagent, counterstained with toluidine blue. Typical calcareous deposits within the sub-mantle connective tissue cells are shown.

D, ovotestis of snail. Ferric substitution method used to reveal calcium deposits. The photograph shows the large number of small calcareous granules present in the cytoplasm of two ova.

E, gut diverticulum of the crab. Ferrous substitution method for calcium, counterstained with eosin. The photograph shows a typical section of the digestive epithelium. Phase-contrast.

F, left colleterial gland tubules of the cockroach. Transverse section showing the single-layered epithelium and the crystals of calcium oxalate in the lumen. KOH / quinalizarin reagent (McGee-Russell, unpublished) was used to colour the calcium deposits.

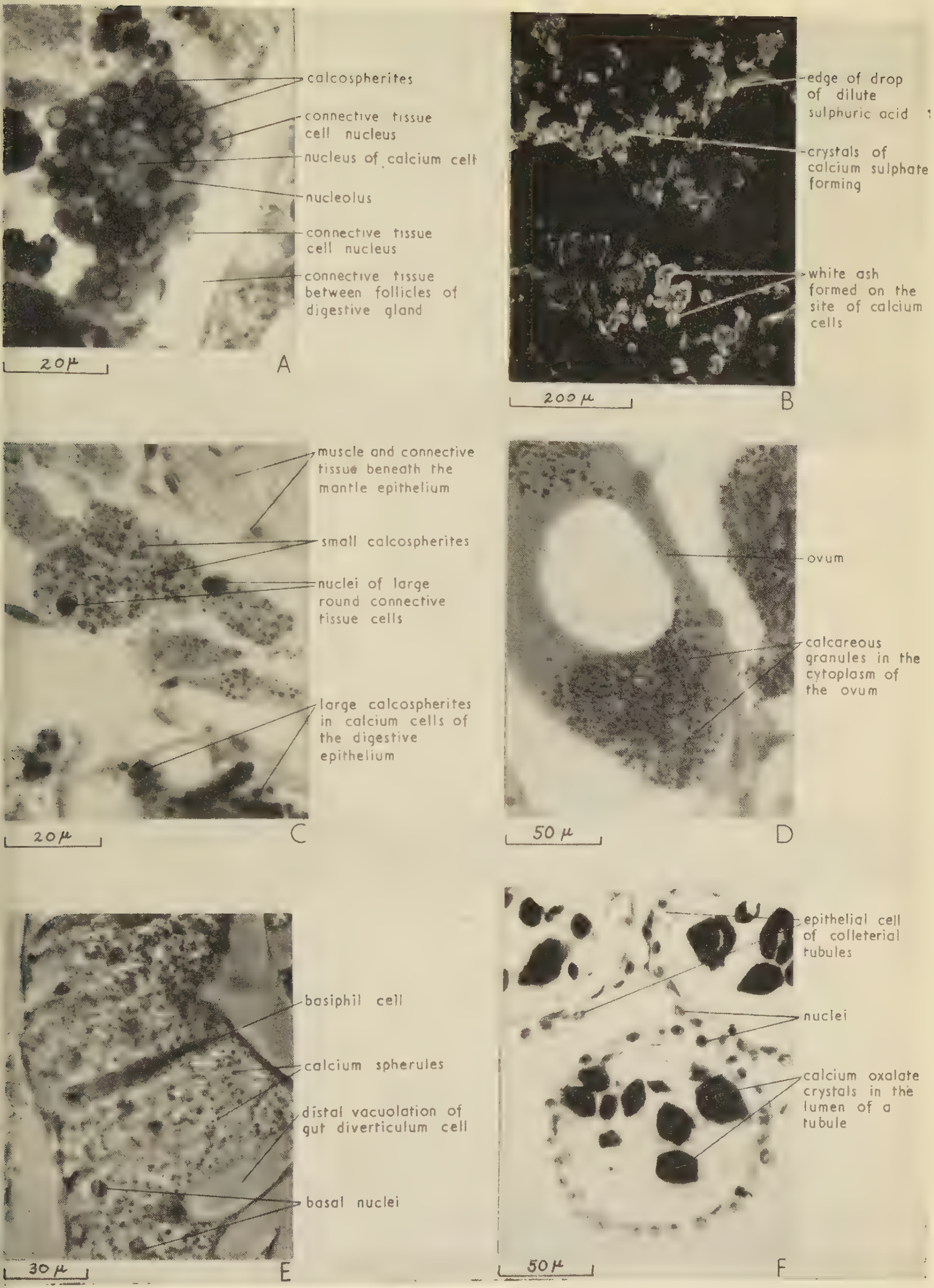
calcospherites
connective tissue cell nucleus
nucleus of calcium cell
nucleolus
connective tissue cell nucleus
connective tissue between follicles of digestive gland
20 μ
A
edge of drop of dilute sulphuric acid
crystals of calcium sulphate forming
white ash formed on the site of calcium cells
200 μ
B
muscle and connective tissue beneath the mantle epithelium
small calcospherites
nuclei of large round connective tissue cells
large calcospherites in calcium cells of the digestive epithelium
20 μ
C
ovum
calcareous granules in the cytoplasm of the ovum
50 μ
D
basiphil cell
calcium spherules
distal vacuolation of gut diverticulum cell
basal nuclei
30 μ
E
epithelial cell of colleterial tubules
nuclei
calcium oxalate crystals in the lumen of a tubule
50 μ
F

Fig. 1

S. M. McGEE-RUSSELL

coloured lilac or violet (Lison, 1953). My tests showed that the colour of the calcium spherules was indistinguishable between control sections deparaffinized in xylene and mounted immediately in balsam, and sections which had been brought to distilled water and treated with warm cobalt nitrate solution. Definitive identification of the crystalline form of the spherules must wait upon crystallographic studies of the spherules, after separation from the tissue by centrifugation.

The positive identification of the spherules as calcium phosphate by authors such as Krijgsman (1928) is puzzling, unless the proportion of carbonate and phosphate varies considerably from one locality to another, perhaps in accordance with diet. An alternative explanation is that the methods of identification used were insufficiently precise. I have been unable to confirm Krijgsman's observation that the addition of ammonium molybdate solution, slightly acidified with nitric acid, to tissue sections, accompanied by gentle warming, produces a yellow precipitate with the spherules. He also found that the spherules dissolve in dilute acids without effervescence. I do not agree. His most demonstrative proof of the apparent phosphate content of the spherules was obtained by boiling the fresh gland for 2 minutes with concentrated nitric acid, filtering, and adding ammonium molybdate solution. A yellow precipitate insoluble in acids but soluble in ammonia was produced. The hydroquinone / molybdate test confirmed the presence of phosphate by a deep blue coloration. The whole tissue, if treated in this way, undoubtedly can be shown to contain phosphate. The radioautographic studies of Fretter (1952) clearly show that the digestive gland is a storage organ for phosphate. Nevertheless, the properties of the snail spherules themselves, and their contrast with the properties of the spherules found in *Carcinus*, convince me that the calcium salt is predominantly the carbonate in the snails which I have studied.

Additional sites of calcium in tissues closely associated with the digestive gland of Helix

It is often possible to find areas of calcareous deposits immediately below the mantle covering the digestive gland, which differ from those in the cells of the digestive epithelium. The deposits are intracellular, in the form of calcospherites which are usually smaller than those of the calcium cells of the digestive gland (fig. 1, c). The structure of the cells containing them is often difficult to make out in fixed preparations, but the cells appear to be the same as the large round cells which are a characteristic component of the connective tissue of molluscs. It is often more convenient to dissect out the whole tip of the visceral hump without removing the overlying mantle, when fixing the digestive gland; in sections of such material the submantle deposits are found. They would appear to correspond to the deposits figured by Manigault in the connective tissue of *Helix* (Manigault, 1939, fig. 47).

The ovotestis lies within the loop of the digestive gland which occupies the tip of the visceral hump. In sections of such material the ova within the ovotestis may show a further site of calcium in the form of small granules within the egg cytoplasm (fig. 1, D).

The gut diverticulum of Carcinus

In fixed preparations the epithelium of the gut diverticulum is found to be composed principally of two types of cell: a columnar cell with vacuolated cytoplasm, throughout the basal region of which a number of calcium spherules is scattered, and a somewhat narrower columnar cell with an intensely basiphil cytoplasm (fig. 1, E). The two types are easily distinguishable in preparations stained with toluidine blue, in consequence of this basiphilia. The cells are approximately 60 μ in height and have a basal nucleus of diameter about 10 μ, which has a well-marked nucleolus and very little chromatin. Further subdivision of the cell types of this epithelium is irrelevant to the present purpose. The cells are extremely fragile, and in teased preparations, mounted under the cover-slip, it is usual to find large numbers of calcium spherules released from shattered cells. In fixed preparations it is noticeable that the number of calcium spherules to each cell is smaller than in the snail tissue, where the entire volume of the cell is packed with spherules. It would appear from the basal distribution of the calcium spherules in the crab cells, and the considerable vacuolation of the distal cytoplasm, that these cells have additional functions other than calcium storage.

The nature of the calcium stores of the 'liver' or digestive gland of the crab does not appear to have been in dispute. Robertson (1937) states that the spherules are largely composed of calcium phosphate, and my findings are in agreement with this statement.

The spherules are completely isotropic. Mounted immediately after removal of the paraffin from sections, they do not show the apparently tinted and refringent appearance of the snail spherules, but are colourless. With dilute mineral acids they do not produce effervescence, but dissolve rapidly. The ammonium molybdate reagent does not produce a noticeable yellow precipitate when applied to sections, but a very heavy positive coloration for phosphate is formed when it is applied to spherules separated out by centrifuging. Application of silver nitrate solution to sections and to the separated spherules results in the rapid formation of a yellow precipitate on and around the spherules; this can only be the iodide or the phosphate. When dilute acid is applied to a very large aggregation of differentially separated spherules, there is rapid dissolution, and occasionally a very slight amount of effervescence, which may indicate that a small quantity of carbonate is present. With dilute sulphuric acid, gypsum crystals are quickly formed. There can be little doubt that the spherules contain a preponderance of calcium phosphate.

The left colleterial gland of Periplaneta

Brunet (1952) has reviewed the literature concerning the identification of the crystals in the lumen of the colleterial tubules (fig. 1, F). There is no disagreement over the identification of them as calcium oxalate by Hallez (1909).

My investigations confirm the presence of calcium in these crystals, and their high insolubility suggests the oxalate salt. Microchemical tests accord with this identification.

FIXATION

In order to study the distribution of calcium in a tissue, fixation must be carried out in non-acid fixatives which do not contain metal salts likely to interfere with the histochemical methods used.

A mixture of absolute alcohol with concentrated (40%) formaldehyde solution (50: 50) was found to be preferable to alcohol alone, for the fixation of the tissues discussed. Alcohol alone produced considerable distortions. The pH of the mixture was measured as somewhat acid (pH 4·7) with a Marconi glass electrode pH meter, but this was not found to disturb the localizations of the calcium deposits mentioned above. Fixation was usually carried out overnight. It is reasonable to adjust the pH of this fixative to neutrality with alkali, if this is felt to be necessary, although it results in no detectable change in the histochemical distribution of the calcium deposits.

A number of carefully controlled comparisons of the results obtained by fixing pieces of the same tissue in alcohol, formaldehyde-alcohol (50: 50), and formaldehyde-calcium (Baker, 1945) showed that the use of the last fixative did not lead to false histochemical distributions of calcium, and that fixation was reasonably good. However, it is very possible that in other tissues there are likely to be sites where calcium might be taken up from this fixative, and the additional controls necessary when using it militate against it.

EMBEDDING

The tissues may be used most satisfactorily if embedded in 58° or 60° C paraffin wax and sectioned at 6 μ. Thin sections are advisable. The tissues may also be embedded successfully in celloidin, ester-wax, 'Aquax' (G. T. Gurr Ltd.), or gelatin. The practice of hardening gelatin blocks in formaldehyde / alum solution was found to be inadvisable, as it tended to remove the calcium salts present. Satisfactory hardening was obtained with 70% alcohol.

My thanks are given to Professor A. C. Hardy, F.R.S., for the facilities he afforded me in his Department, and to Dr. J. R. Baker and Dr. P. C. J. Brunet for much encouragement. I am grateful for the scholarship awarded me by the Christopher Welch Trustees, and for the additional financial assistance of the Department of Scientific and Industrial Research.

REFERENCES

Baker, J. R., 1945. Quart. J. micr. Sci., **85**, 1.
Barfurth, D., 1880. Zool. Anz., **3**, 499.
Brunet, P. C. J., 1952. Quart. J. micr. Sci., **93**, 47.
Cameron, G. R., 1930. J. Path. Bact., **33**, 929.
Cretin, A., 1923. Thèse pour le Doctorat en Médecine, Paris, No. 253. Faculté de Médecine.
Fretter, V., 1952. Quart. J. micr. Sci., **92**, 133.
Hallez, P., 1909. C.R. Acad. Sci. Paris, **148**, 317.

HEAYSMAN, J. E. M., 1951. Ph.D. thesis, University of London.
KEILIN, D., 1921. Quart. J. micr. Sci., **65**, 611.
KRIJGSMAN, B. J., 1928. Z. vergl. Physiol., **8**, 187.
LISON, L., 1936. *Histochimie animale,* 1st edition. Paris (Gauthier-Villars).
—— 1953. 2nd edition.
MANIGAULT, P., 1936–7. Ann. Sci. nat., **19-20**, 55.
—— 1939. Ann. Inst. océanogr. Monaco, **18**, 33.
MCGEE-RUSSELL, S. M., 1954. D.Phil. thesis, University of Oxford.
PRENANT, M., 1927. Biol. Rev., **2**, 365.
ROBERTSON, J. D., 1937. Proc. Roy. Soc. B, **124**, 162.
—— 1941. Biol. Rev., **16**, 106.
SIOLI, H., 1935. Zool. Jb. allg. zool., **54**, 507.
STOCK, A., 1949. J. Roy. micr. Soc., **69**, 20.
WAGGE, L. E., 1948. Ph.D. thesis, University of London.

A Method for High-power Stereoscopic Microscopy

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SUMMARY

An expanded account is given of a simple modification to an ordinary beam-splitting microscope, which renders it stereoscopic. The full aperture of the objective is used, so the method is applicable to all powers of the microscope. The method involves dividing the lower focal plane of the condenser into left and right halves with opposite polarization by means of Polaroid, and isolating the two resulting beams in the eyepieces with Polaroid analysers.

This system, first published by Jackson (1944–9), gives excellent results with the highest powers of the microscope, but appears to have been forgotten. This paper is written in the hope of re-introducing it.

INTRODUCTION

WHEN an object is viewed with both eyes, each receives a slightly different picture on the retina. These two views are fused into one by the brain, and the resulting composite view gives a strong impression of solidity and depth, dependent on the minute discrepancies in the two views. This appearance of depth can be duplicated by any instrument, such as binoculars or a stereoscope, which presents suitable slightly different views to the two eyes.

In microscopy, it is often important to determine the structure of an object in depth, so that stereoscopic microscopes would be valuable. It must be appreciated that, whereas all stereoscopic microscopes are necessarily binocular, not all binocular instruments are stereoscopic, as will be seen.

Binocular microscopes are of three main types:

1. *Twin microscopes.* These have two objectives as well as two eyepieces, giving excellent stereoscopy, but the two objectives limit them to low powers. Dissecting microscopes are of this type, with right-angled prisms in each tube to erect the image and provide adjustment for the inter-ocular spacing.

2. *Beam-splitters.* These have only one objective, the light from which is divided into two identical parts by the half-silvered surface of a cubic prism, or a similar arrangement. Both eyes receive the same view exactly, so the microscope is not stereoscopic. It is intended purely to relieve eyestrain during prolonged observation. The method is applicable to any power of objective or eyepiece.

3. *Wenham prisms and variations.* These also have only one objective, but prisms are arranged in the back focal plane of the objective so that the light from the left half of the back lens goes to the right eyepiece and conversely. Therefore true stereoscopy results, but at the expense of resolution, as the aperture of the objective is halved. It is relevant to point out that the stereoscopic microscopes which work by employing a beam-splitter and placing

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opaque semicircular stops above the eyepieces in the Ramsden circles may be regarded as belonging to this class of binocular, and the same remarks about aperture apply. This is because optically there is no difference between stopping the Ramsden circle and stopping the back focal plane of the objective, since these are conjugate planes. Owing to the drop in resolution due to the decrease in the aperture of the objective, these systems are not suitable for the highest powers of the microscope.

It can be seen, therefore, that there is no well-known microscope giving true stereoscopic vision at high resolution. The purpose of this paper is to re-introduce a way of filling this gap.

METHOD

This method was suggested in a short paper by Jackson (1944–9), which unfortunately contained little detail and no explanations. The following is an expansion of his account with added remarks of my own.

The apparatus is a simple modification of a beam-splitting microscope—that is, one in which both eyes see identical views, obtained with the full objective aperture. Polaroid is inserted in such a way that the full objective aperture is used, only the condenser aperture being halved. As is known (Baker, 1952), this has a much less deleterious effect on resolution than halving the objective aperture, as in the Wenham systems.

A disk of Polaroid is cut, of the correct size to fit into the substage filter-holder. A line is scratched lightly diametrically across the disk and parallel to its plane of vibration. This may be determined by crossing it with a Nicol or a marked piece of Polaroid. The disk is then cut diametrically across, at an angle of 45° to the plane of vibration. One half is turned upside down and the Polaroid is mounted dry between disks of glass and bound around the edge with adhesive tape. The two halves will then be polarized at right angles to each other, and at 45° to the junction line. Alternatively, the disk may be constructed to give vertical polarization in one half, and horizontal in the other. In this case, one of the two possible orientations of the disk in the microscope will probably give better results than the other.

The binocular beam-splitting microscope is set up in the normal way, with a powerful light source (an opal bulb is inadequate). I find that a Pointolite gives excellent results. The Polaroid disk is then placed in the filter-holder immediately below the condenser, in such a position that it is divided into left and right halves of opposite polarization. This is checked by observing the back lens of the objective: if the two halves are not equal, the disk is not centred, and this must be rectified.

Two Polaroid analysers are placed in the two eyepieces, preferably just above the field lens, so that specks on them will be out of focus. The Ramsden circles above each eyepiece are now examined, either by drawing the head well back from the microscope, or with a hand-lens. They are conjugate with the back focal plane of the objective and the lower focal plane of the condenser, and an image of the polarizer is seen: each Ramsden circle is therefore divided into left and right halves. The eyepieces, with analysers, are rotated until the right half of the left Ramsden circle and the left half of the right circle—that is, the inside halves––are extinguished (fig. 1, A). True stereoscopy then results, which may be checked by comparing the views of the two eyes. If the analysers are rotated so as to extinguish the outside halves of the Ramsden circles instead (B), pseudoscopy or reversed stereoscopy is obtained; while extinguishing both left (C) or both right halves, or equalizing the intensity of the two halves of each disk (D), gives non-stereoscopic binocular vision.

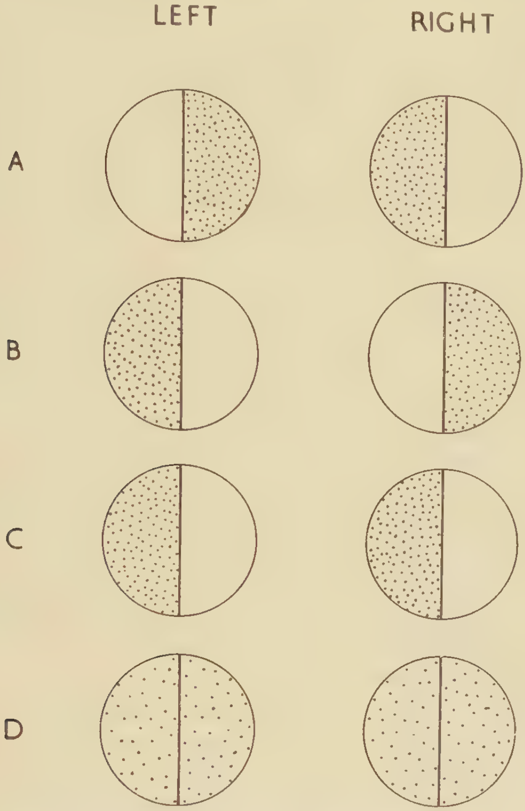

FIG. 1. Diagram showing the appearance of the right and left Ramsden circles above the eyepieces, with four different settings of the analysers.

The method is applicable to any power of the microscope, and objectives can be changed without any altering or resetting. In fact, it has been found that the 2-mm lens gives the most startling results, no doubt owing to the great angular difference between the two direct light beams. It is thought that the failure of the method to achieve popularity in the past was due to its being tried only with low-power objectives, with which its performance is much inferior to a Wenham prism. The advantage of the method lies in the strong stereoscopic effect produced with a 2-mm objective.

Theoretical Considerations

Light normally strikes a microscopical preparation as a cone, symmetrical about the optical axis. If the light is made oblique, e.g. by moving a substage stop, the object appears to change its position and angle to the optical axis by an amount proportional to the obliquity of the light. This must be due to a progressive change in the phase of the direct light from one side of the object to the other, therefore modifying the phases of the various components of the diffracted light in the back focal plane of the objective, and so altering the image. The divided polarizer provides two oblique half-cones of light which can be isolated by the analysers, and also two sets of diffracted light, each of full aperture since the objective is unobstructed, which can be similarly isolated.

The Ramsden circles confirm this—in each can be seen the bright half, which is the direct light of half aperture, together with half of the diffracted light. The dark half of the Ramsden circle is not completely black, partly because of imperfections in the Polaroid, but also because it contains the other half of the light diffracted by the object, and having the same polarization as the direct light. Each eye therefore receives direct light of half aperture, together with diffracted light of full aperture. If the object is strongly diffracting, it will be found impossible to darken half of the Ramsden circle because of the intensity of the diffracted light, and it will be necessary to move the object out of the field of view before the eyepiece analysers can be set.

It should be mentioned that this method cannot be used where the object is strongly birefringent, though slight birefringence, as in muscle, seems to make no appreciable difference.

Depolarization by reflexion in the binocular prisms is not serious in any system that I have tried out, as a very complete extinction is not necessary, provided that the unwanted beam is small in intensity compared with the wanted one. However, some prism systems cause rotation of the plane of vibration, so the setting of the analysers is not necessarily the one expected. This was not remarked upon by Jackson, and is the reason for setting the analysers by observing the Ramsden circles.

Phase contrast can be used with this method of stereoscopy, although a very intense light source is required, and the stereoscopic effect is not so pronounced, because the depth in which the phase effect is produced is smaller than the depth in which out of focus detail can be appreciated stereoscopically. The stereoscopic effect of the focused detail is just as great with phase contrast as with direct microscopy.

I have also successfully applied patch stop dark ground illumination.

I am indebted to Dr. J. R. Baker and Dr. S. M. McGee-Russell for a most interesting discussion of stereoscopy, to Mr. T. A. Minns of Messrs. W.

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REFERENCES

Baker, J. R., 1952. Quart. J. micr. Sci., **93**, 375.
Jackson, J. J., 1944–9. Journ. Quekett micr. Club, 289.

Osmichrome Fixation

By HERBERT ELFTMAN

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With one plate (fig. 1)

SUMMARY

Fixation by the combined action of osmium tetroxide and dichromate involves the use of two reagents which produce their best results at two different hydrogen ion concentrations. By applying them in sequence the conditions may be adjusted independently for the two reagents. For tissues of the rat the following schedule has provided good results: (1) Fix for 5 to 7 hours at 5° C in 1% osmium tetroxide buffered at pH 7·2; (2) rinse briefly; (3) chromate for 18 hours at 20° C in 2·5% potassium dichromate dissolved in 5% mercuric chloride; (4) embed in paraffin.

INTRODUCTION

FAITHFUL preservation of the general framework of cytoplasm has won for osmium tetroxide pre-eminence as a cytological fixative. In the preparation of tissues for study with the light microscope the most common procedures, such as those of Flemming, Altmann, and Champy, have combined the action of osmium with that of chromium in a single solution of marked acidity. The discovery of Palade (1952) that the optimum pH for fixation by osmium is 7·2 or even higher, places the favourable range for its activity well above that in which chromate solutions have their maximum effectiveness (Elftman, 1954).

In order to allow both osmium and chromium to act under congenial circumstances we have experimented with their use in sequence. Besides providing each reagent with more favourable conditions, there is an advantage in being able to experiment with the variables separately. We shall refer to the sequence of initial fixation in an essentially neutral osmium tetroxide solution, with post-chromation in a solution of controlled acidity, as 'osmichrome fixation'.

We shall consider first the osmium reagent, then the chromate reagent, and finally the results achieved by their application.

THE OSMIC REAGENT

The osmic reagent employed in osmichrome fixation is essentially the one recommended by Palade (1952) for the fixation of tissue destined for study by the electron microscope. A 1% solution of osmium tetroxide is prepared in M/40 Michaelis verónal buffer and adjusted to pH 7·2

Although the strength of the osmic solution is stated conveniently in terms of the amount of osmium tetroxide used in its preparation, this should not lead to the assumption that the tetroxide persists in unaltered form in the solution. When osmium tetroxide is dissolved it does not remain aloof but joins with water to form H_2OsO_5 (Yost and White, 1928). This may be called perperosmic acid, but the ionization constant is so small (8×10^{-13}) that some biologists consider the name of acid inappropriate. No misunderstanding

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will result if cytologists continue to refer to solutions of osmium tetroxide, and this usage will be followed in the present paper.

The length of time during which tissues may be subjected to the osmium reagent is governed by two factors. The first is the slowness of penetration. Not only does this limit the size of the block which can be fixed by immersion but it ensures the presence of a fixation gradient, the interior showing some signs of belated access. The second difficulty is the brittleness produced by osmium tetroxide. These difficulties may be minimized by administering the fixative by perfusion. We have found it more practical to prepare our tissue in thin slices, thus decreasing the distance through which diffusion must occur, and to leave them in the 1% osmium tetroxide solution for from 5 to 7 hours in a refrigerator at 5° C. Lowering the temperature slows down the chemical action of the osmium reagent more than it does its diffusion.

On the way to the chromating solution the tissue is rinsed gently to prevent the carrying over of an excess of osmium tetroxide. In our experience no good purpose is served by protracted washing at this stage.

The Chromate Reagent

The second step in osmichrome fixation is chromation, effected by a solution which is conveniently prepared from potassium dichromate, since potassium is a relatively innocuous cation and the acidity of the solution is close enough to the useful range to allow ready adjustment. We have found 2·5% potassium dichromate a useful concentration.

In a previous paper (Elftman, 1954) attention was called to the fact that the oxidative action of hexavalent chromium is particularly responsive to hydrogen ion concentration but is also accelerated by rise in temperature and is cumulative with time. There is consequently no unique combination of pH, temperature, and time to be prescribed for chromation. For routine use we prefer to restrict the chromation time to 18 hours; this allows tissues collected one morning to be osmicated during the day, chromated overnight, and embedded the following morning. The decision as to which temperature to employ depends to a certain extent on the tissue under study. Less physical distortion occurs when the temperature is not unduly elevated; for most purposes a room temperature of 20° C suffices.

With time and temperature specified, acidity is no longer a free variable. Our best results have been achieved with the chromating solution acting at a pH of 2·5–3·0. In order to stabilize the acidity without introducing deleterious ions we have employed mercuric chloride. The chromating solution which we use most frequently at present is prepared by dissolving potassium dichromate at 2·5% in a stock 5% solution of mercuric chloride. The pH of this solution may be adjusted to 2·5 by the addition of hydrochloric acid to accelerate the early stages of oxidation, or it may be used with its unmodified initial pH of 3·0. In either case the terminal pH after 18 hours of contact with the osmicated tissue is usually about 3·5, a rise considerably greater than that which occurs with fresh tissue during controlled chromation.

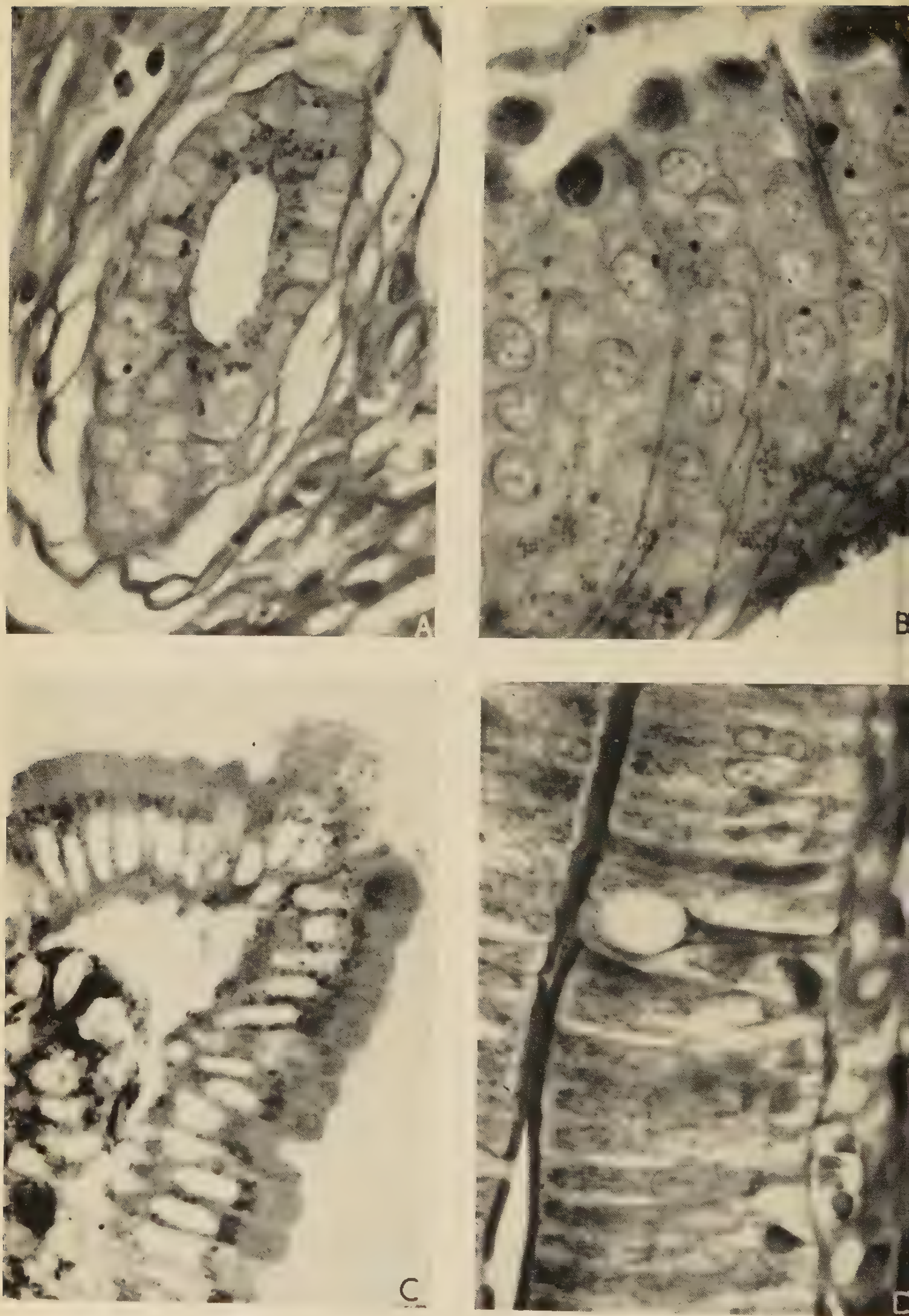

Fig. 1

H. ELFTMAN

The preparation of osmichrome-fixed tissue for embedding follows the usual routine. Of the numerous clearing agents which are available, methyl benzoate (oil of Niobe) deserves commendation. For the small blocks of tissue currently of interest in this laboratory immersion in methyl benzoate for an hour and a half at 37° C is sufficient. Its relatively rapid action and constancy of composition are points of superiority over cedar oil. The subsequent infiltration with paraffin should not be prolonged unnecessarily. Sections may be cut readily at $2\,\mu$.

RESULTS

Characteristic results obtained with osmichrome fixation may be studied in the photomicrographs, fig. 1, A–D. We have been able to utilize the usual range of staining techniques with a minimum of the difficulty so frequently associated with osmic fixation.

The periodic acid / Schiff procedure has resulted in the staining of granules of the Golgi apparatus in the uterus (fig. 1, A). This should not be taken as evidence of the presence of carbohydrate, since oxidation of unsaturated bonds of phospholipids provides a precursor for this reaction and osmium itself can affect the reagent.

Altmann's anilin / acid fuchsin not only stains the mitochondria of the intestine (fig. 1, D) but also provides a clear presentation of the centrioles of the testis (fig. 1, B). The acroblasts are faintly visible in this section; they may be stained more deeply by the periodic acid / Schiff method.

The use of Sudan black is illustrated by the villus of fig. 1, C. The Golgi lipid is deeply stained and the apical mitochondria have taken up more of the dye than have the basal ones. Variation of the staining reaction with distance in from the tip of the villus follows a regular sequence and may be indicative of changes in the cells as they progress towards the apex. A comparison of Sudan black staining of osmichrome tissue with that treated by controlled chromation reveals a difference between basal and apical mitochondria in their reaction to osmium tetroxide.

DISCUSSION

In order to interpret the results of osmichrome fixation, some consideration must be given to the method of action of the reagents. The chemistry of

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FIG. 1 (plate). Tissues of Long–Evans rats after osmichrome fixation. Sections $5\,\mu$ thick. Photomicrographs taken on Kodak Commercial Ortho film with a Wratten 60 filter.

A, uterine gland, stained with the periodic acid / Schiff procedure, showing a positive reaction of the Golgi material.

B, testis, stained with Altmann's anilin / acid fuchsin and methyl blue. The acid fuchsin is concentrated in the centrioles. The acroblasts are more faintly visible.

C, tip of villus of small intestine, stained with Sudan black in ethylene glycol. The clear oval spaces are nuclei (no nuclear stain was used). The Golgi apparatus and the mitochondria are evident.

D, epithelium of small intestine, stained with Altmann's anilin / acid fuchsin and methyl blue. Threadlike apical mitochondria and more rounded basal ones may be seen. In the lower portion of the section the Golgi apparatus has also taken up the acid-fuchsin.

fixation by osmium has been reviewed succinctly by Baker (1945) and in detail by Berg (1927). More recent experiments have been described by Porter and Kallman (1953) and by Bahr (1954). The physical results of the action of osmium on proteins may be described as gelation without coagulation.

The action of osmium tetroxide on lipids has caused considerable perplexity since unsaturated triglycerides will blacken rapidly while phospholipids require prolonged treatment before final reduction of the osmium is accomplished. An explanation suggested by the work of Criegee (1936, 1942) envisages a two-stage process. The first stage results in an ester which, though not black to transmitted light, will be opaque to electrons and therefore be useful in electron microscopy. The second stage, reduction of the osmium to the dioxide or the metal, is not always a simple matter. Empirical methods of accomplishing this second reaction may be found in the numerous osmic procedures for the Golgi apparatus.

With fixation of proteins accomplished by osmium tetroxide, the particular function which remains to be performed by the chromating reagent is concerned with the phospholipids. They must be rendered insoluble in the reagents to be applied later and left in a chemical state which will allow analytical procedures to be performed upon them. In osmichrome fixation the action of the hexavalent chromium is influenced to a certain extent by the first-stage reaction of osmium tetroxide with the phospholipids.

Osmichrome fixation adds one more to the long list of procedures in which osmium tetroxide and dichromate have worked in partnership. It is hoped that the separation of variables made possible by applying the reagents in sequence will facilitate further experimentation with problems of fixation.

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REFERENCES

Bahr, G. F., 1954. Exp. cell Res., **7**, 457.

Baker, J. R., 1945. *Cytological technique.* 2nd ed. London (Methuen).

Berg, W., 1927. In *Enzyklopädie der mikroskopischen Technik*, edited by R. Krause. 3rd ed., vol. 3. Berlin (Urban und Schwarzenberg).

Criegee, R., 1936. Liebig's Annalen der Chemie, **522**, 75.

—— Marchand, B., and Wannowius, H., 1942. Ibid., **550**, 99.

Elftman, H., 1954. J. Histochem. and Cytochem., **2**, 1.

Palade, G. E., 1952. J. exp. Med., **95**, 285.

Porter, K. R., and Kallman, F., 1953. Exp. cell Res., **4**, 127.

Yost, D. M., and White, R. J., 1928. J. Amer. chem. Soc., **50**, 81.

A Histochemical Study of the Organic Matrix of Hen Egg-Shells

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SUMMARY

A study of the organic material of the hen egg-shell has been made by the use of recognized histochemical techniques.

It has been found that, in the so-called spongy layer of the shell, the organic matrix consists of a protein / acid mucopolysaccharide complex. The organic material of the mammillary layer appears to be more concentrated in a central core, and is somewhat different in that it contains fat and a reducing substance.

The protein / acid mucopolysaccharide complex is a collagen-like material, but is probably not collagen itself because the protein portion is low in hydroxyproline and the acid mucopolysaccharide is most likely mucoitin and not chondroitin sulphuric acid.

The results are discussed in relation to shell calcification and it is suggested that the protein / acid mucopolysaccharide complex is first firmly attached to the keratin of the membrane, but once this is accomplished it links on to material of its own kind. Calcification proceeds simultaneously, but near the points of contact with the membrane (i.e. in the core of each mammilla) calcification is not so intense as it is in the rest of the shell.

INTRODUCTION

ALTHOUGH the hen egg-shell is of fundamental importance both in the commercial preservation and marketing of eggs, and also in the development of the incubated chick, little work has been done on shell structure.

Stewart (1935) gave a good general review of what was known of the structure of the hen egg-shell at that time. He recognized the two shell membranes which lie over the albumen of the egg and which are composed of a network of fibres. Attaching the outer membrane to the shell are the mammillae, which Stewart described as knobs composed of large spherulite crystals of calcite. These knobs have organic cores and are roughly hemispherical, being continuous above with the main body of the shell, which is referred to as the spongy layer. This is badly named because it is composed of calcite crystals bound together by a sparse organic matrix. The shell is covered by a cuticle or 'bloom' which also covers the mouths of the pores. The pores are funnel-shaped tubes, round or oval in cross-section. They start from the interstices between the mammillae, pass through the main body of the shell, and finally open under the cuticle (fig. 1). More recently, Tyler (1956) has studied certain aspects of shell structure, including pore size and shape, by using plastic embedding techniques.

Moran and Hale (1936) investigated the structure of the two shell membranes, which they were able to divide into several layers The outer mem-

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brane, i.e. the one next to the shell, is composed of three layers, the outermost consisting of keratin fibres 2–5 μ in diameter and the other two of mucin fibres 0·8 μ in diameter. The inner membrane, which lies immediately over the egg albumen and forms the inner boundary of the air space, is composed of two rather indistinct layers of finer keratin and mucin fibres. The outer membrane is the thicker of the two. According to Calverly (1933) both membranes are composed mainly of keratin fibres.

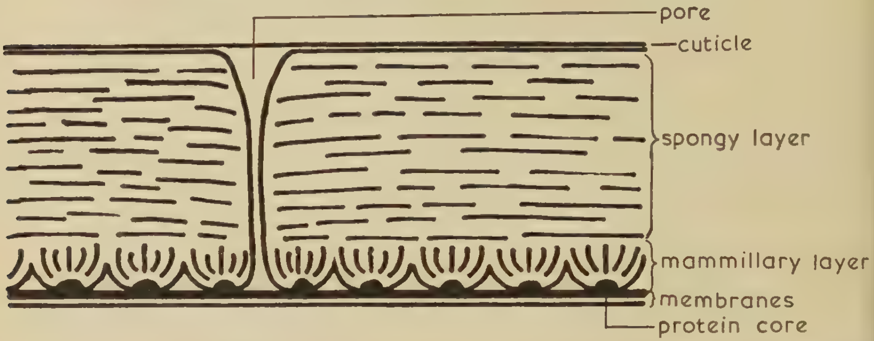

FIG. 1. Conventional drawing of a radial section of an egg-shell, showing mammillae with protein core, spongy layer, membranes, and cuticle. The section also shows a pore.

Little work has been done on the organic matrix which binds the calcite crystals of the shell together, except that Almquist (1934) has stated, on the basis of a chemical investigation, that this protein differs from the membrane protein and may be classified as a collagen-like substance.

Dickie (1848) originally suggested that the cuticle was a cellular epithelium over a basement membrane. This view has never been directly attacked, but Marshall and Cruickshank (1938) stated that they considered the cuticle to be a somewhat porous but apparently structureless substance. These authors also introduced the concept of 'cuticular plaques', which are the thickenings of the cuticle over the mouths of the pores; they further suggested that these regions of the cuticle might act as an additional evaporating surface for the removal of water vapour from the egg. Moran and Hale (1936) and Haines and Moran (1940) considered the cuticle to be mainly mucin.

Tyler and Geake (1953) treated shells with a boiling 2·5% sodium hydroxide solution for 5 min and showed that this removed the membranes cleanly and probably some of the cuticle. Further treatment with 10% sodium hydroxide solution led to the removal of more protein, but after about 7 hours no more protein could be removed. They considered that the resistant protein was matrix protein, mechanically beyond the attack of the solvent, and that the portion removed was probably pore protein present in the pore channels.

It was felt that much remains to be done on the proteins of the shell and it was decided to use the simple staining and more complex histochemical techniques as a start. This paper describes work on the matrix and mammillae.

METHODS

Decalcification

Shells were decalcified with solutions of the sodium salts of ethylene diamine tetra-acetic acid (EDTA). The original method was to use an 8% solution of the disodium salt in water (pH 4·3–5·3) which had been brought to pH 7 with either sodium hydroxide or sodium bicarbonate. More recently, a 6% solution of the trisodium salt has been used without further treatment. This solution has a pH of about 8.

The use of a chelating agent in a slightly alkaline solution eliminates the possibility of carbon dioxide bubble formation during decalcification. This is a great advantage because the ease with which carbon dioxide is produced during acid decalcification of shells probably explains the damage to the organic structure and consequently the poor results obtained by previous workers.

Formalin was added to the decalcifying solution to a strength of 6% so that fixation would accompany, if not precede, the decalcification. Used in this way any acidity in the formalin solution was neutralized by the EDTA solution and hence did not produce bubbles of carbon dioxide from the shell. Formalin was chosen as the fixative because (*a*) it can be used in an approximately neutral solution, (*b*) it is not dependent on the presence of metallic ions which the EDTA would remove, (*c*) it is of wide general application both histologically and histochemically, and (*d*) its mechanism of fixation is at least partially understood.

Shells decalcified in this way were washed in water, dehydrated in a series of alcohols, cleared in benzene, and embedded in 52°–56° C paraffin wax. Sections were then cut at 5–10 μ for routine work.

Histological and histochemical techniques

The sections were originally stained with Mallory's triple stain and from the general structure revealed it was then possible to use more specific histochemical methods. As the work progressed the methods became centred around tests for three main types of substance, namely, mucin, protein, and fat. The methods used have been based chiefly on Pearse (1953), but references to original work are given where necessary.

Mucins. These have been investigated by using such stains as mucicarmine, toluidine blue, and alcian blue (Steedman, 1950). Hale's (1946) dialysed iron method, though less specific, was also used. The periodic acid / Schiff test (PAS) of McManus (1948) has also been used in this study and also the modification employing acetylation before the test (McManus and Cason, 1950).

The effect of calcium ions on the metachromatism produced by toluidine blue has also been studied. This technique was first described by Sobel and Hanok (1952) and Sobel and Burger (1954).

Proteins. Millon's reagent was used as a test for 'phenolic' proteins (Pollister's modification as quoted by Gomori (1952)), but a more satisfactory resul has been obtained by using the tetrazotized benzidine-β naphthol (TBN method of Danielli (1947).

Fats. Sudan black B in propylene glycol (Chiffelle and Putt, 1951) or in 70% alcohol, Sudan III, Fettrot red 7 B (Ciba), and Nile blue sulphate (Cain 1947) have been used along with the performic acid / Schiff test (PFAS) of Pearse (1951). Since this last test gives a positive result with either keratin or unsaturated fats it was necessary to use it in conjunction with other tests such as control slides stained with Sudan black B or the use of methylene blue in veronal acetate buffer at pH 2·6 after performic acid treatment. The former will detect fat and the latter will distinguish between the oxidation-products of the two kinds of material.

The above methods for fat have been employed before and after attempting the extraction of fat from sections. The attempt was made by treating consecutively with cold acetone, hot acetone, hot ether, and hot chloroform / alcohol (24 h in each), or alternatively pyridine at 60° C for 24 h.

Enzyme treatments. Hyaluronidase (Benger's 'hyalase') has been used in a 1 mg/ml solution in 0·85% saline in an attempt to 'digest' mucins. Pepsin and trypsin solutions were also tried with the appropriate tests applied before and after the enzyme treatment. Crystalline pepsin was used at a strength of 2 mg/ml in 0·02 N.HCl to give a pH of 1·6, and the slides were incubated for 2–3 h at 37° C. Purified trypsin was used at 0·1 mg/ml in 0·03 M phosphate buffer at pH 6·0, and the slides were incubated at 37° for 15–60 min.

Diastase has also been used in 1% aqueous solution for 30 min at room temperature as a test for glycogen.

Basiphilia. The methylene blue extinction test (MBE) was used over a range of pH values.

Reducing compounds. The ferri-ferricyanide test of Chevrement and Frederic (1943) was used, being tried before and after the blocking of sulphydryl groups with phenyl mercuric chloride in butanol.

Vital staining

A laying hen was injected with 5 ml of a 1·25% solution of Evans blue stain. This stain was forced about 10 cm. up the cloaca and oviduct of the hen with a syringe about 4 h after laying. A second hen was similarly treated. In both cases much of the stain was forced out by peristalsis when the syringe was removed, but sufficient almost certainly remained in the tract. Each hen laid the next day at the expected hour and the shells were examined.

The hen used in the first experiment also had 10 ml Evans blue stain injected intravenously into each wing vein; again this was done 4 h after an egg had been laid and the bird laid next day as expected.

It is of interest to note that 3 weeks after these treatments the hens were killed and the liver, ovaries, oviduct, and cloaca were well stained, indicating that the injections had reached their objective.

RESULTS

Matrix

The TBN reaction showed clearly that the matrix contains some protein; other tests indicated mucin substances, whilst the diastase test showed glycogen to be absent.

The matrix stained red with mucicarmine, indicating that the blue staining with Mallory's triple stain was caused by a mucin substance, whilst Hale's dialysed iron method gave a positive result, suggesting acid mucopolysaccharide; alcian blue gave a result strongly supporting this. The degree of basiphilia was tested by the MBE test. Sections left in the solution for 24 h and viewed without dehydration showed that the matrix could bind the dye at pH 5, though it was usually unable to do so at pH 4. This suggests that the material is acidic.

The PAS test was strongly positive and furthermore the reaction was blocked by a prior treatment of the sections with acetic anhydride. The effect of acetylation was then removed by alkali and the test became positive once more. This indicates that two hydroxyl groups or one hydroxyl and one amino group are present on adjacent carbon atoms, possibly as carbohydrate residues, in the matrix. Toluidine blue staining was also tried on the acetylated material and the metachromatism was unaffected. It would thus appear that the two tests detect different aspects of the same molecule, the PAS test detecting the groups on adjacent carbon atoms and the toluidine blue the polymerized and/or acidic nature of the molecule. Direct toluidine blue staining either in very dilute (0·001%) or in acidic buffered (pH 3·6) solutions at 0·01% showed that the matrix was strongly γ-metachromatic.

Sobel and his co-workers (Sobel and Burger, 1954; Sobel and Hanok, 1952) investigated the effect of calcium ions on the metachromatism of rachitic bone, part of which had not been calcified. The present writers had, naturally, to be content with decalcified shells. In the first experiment with formalin-fixed paraffin sections, solutions of 0·01% toluidine blue in glass-distilled water gave, with various concentrations of calcium, the following degrees of metachromatism as assessed visually:

Ca^{++} *M.eq. per litre*	*Metachromatism*
0	++++
5	++++
20	++++
40	++
83	+
250	Trace
500	0

Staining a similar section in toluidine blue containing 250 m.eq. per litre sodium ions had no effect on the metachromatism (i.e. ++++), but when the slide was transferred to a solution with 250 m.eq. per litre calcium ions the metachromatism disappeared. These results are almost identical with those obtained by Sobel, and indicate competition between toluidine blue and calcium ions for combination with the matrix substance. The experiments were repeated with unfixed frozen gelatine-embedded material, instead of formalin-fixed paraffin sections, but substantially the same results were obtained. Thus the decalcified unfixed material gives the same results as those reported by Sobel and Burger (1954) for fixed rachitic bone. The process of calcification of the shell in itself may therefore produce some change in the matrix.

A further experiment was tried with formalin-fixed paraffin sections which had been digested for 3 h with pepsin. These sections showed only a faint metachromatism and it was thought that the protein may have been removed, causing the liberated mucopolysaccharide to undergo some degree of depolymerization and hence to show a low level of metachromatism. To follow up this point toluidine blue with various concentrations of calcium was tried on slides after pepsin treatment. There was, however, no increase in metachromatism, but only a falling off at 100 m.eq. per litre as previously. It would appear, therefore, that the reduced metachromatism found is caused by a small part of the matrix which has not been completely digested, i.e. most of the protein is removed and the liberated mucopolysaccharide lost in the solution. This was confirmed by the PAS and TBN tests on sections digested by pepsin.

When trypsin was tried on formalin-fixed paraffin sections, no effect was shown structurally or in their response to toluidine blue. Hyaluronidase was then tried for 3 h and the result was similar to that for trypsin; however, there was possibly some reduction in metachromatism after 18 h. It was found in other experiments with formalin-fixed paraffin sections after 3 h in hyaluronidase that if a slide was stained in dilute toluidine blue (0·01%) and viewed immediately it showed no metachromatism, but if left for 30–60 min the full colour developed. This was further investigated by placing slides treated with hyaluronidase either in glass-distilled water, or in a solution of 15 m.eq. per litre calcium ions for 1 h. The slides were then washed and stained simultaneously in 0·005% toluidine blue, metachromatism being scored against time. The following results were obtained as means of three experiments:

Time (min)	*Treatment*	
	Water	Ca *solution*
1	−ve	½+
3	−ve	++
5	−ve	++++
10	+	++++
15	+	++++
20	++	++++
30	++++	++++

These results are similar in their interpretation to those obtained by Sobel and Burger (1954) for unfixed rachitic bone. It appears that formalin fixation does not stop depolymerization by hyaluronidase, but anchors the depolymerized mucopolysaccharide on to the protein remaining on the slide. The depolymerized mucopolysaccharide is then easily repolymerized by treatment with calcium ions. Toluidine blue also does this, but much more slowly at the concentration used in this experiment.

Mammillae

The mammillary 'knobs' are essentially the inner part of the shell and in its staining properties their organic material is very similar to the organic matter of the matrix with which it is continuous. Thus the mammillae stained with Hale's dialysed iron and mucicarmine and gave a stronger PAS reaction than the main part of the matrix. They differed from the matrix in that toluidine blue stained orthochromatically and Mallory's triple stain showed a red core within the base of each one. Staining with Sudan black B showed that there is a large amount of fat present in the mammillae, whereas the matrix only stains very faintly.

The ferri-ferricyanide test showed that the mammillae contain some compound which gives a positive reaction. This test was then repeated on alcohol-fixed material with the same result. Furthermore, the reaction was not prevented by a previous treatment of up to 14 days with phenyl mercuric chloride in butanol, which indicates that sulphydryl (-SH) groups were not responsible for the reaction. Substances, other than sulphydryl groups, which are capable of reducing ferricyanides are therefore present in the mammillae. The matrix itself did not give a positive result with the ferri-ferricyanide test.

The TBN reaction was strongly positive in the mammillae, showing these to be rich in protein, but the matrix itself gave a weaker reaction. This was confirmed with Millon's reagent.

The mammillae, therefore, although part of the shell, give some reactions which indicate that they contain organic compounds which are different from those in the rest of the shell.

Gersh and Catchpole (1949) have expressed certain ideas on the relationship between polysaccharide complexes and reactions with calcium. They consider that water-soluble unpolymerized acid mucopolysaccharides would copolymerize with calcium, and were important in calcification. This unpolymerized material was also more reactive towards the PAS test. It was, therefore, decided to investigate this point with regard to the mammillae, which are probably the first sites of shell calcification. Shells were decalcified for several days in aqueous EDTA solutions without any fixative. Water-soluble components were thus given ample time to dissolve and diffuse away, provided they were not affected by the EDTA. This material was then dehydrated, sectioned from paraffin blocks, and compared with alcohol and formaldehyde EDTA decalcified material from the same shell. The material was examined in tangential section so that the mammillary region could be studied over a greater area. Mallory's triple stain was used on all these sections and the aqueous decalcified unfixed material showed no loss by solution of either the red core or the blue outer covering of the mammillae.

In the vital staining experiments it was hoped that depolymerized polysaccharide material would show up if present. Thus, if it is in the blood, the intravenous injection should have caused the dye to be deposited in the shell. If, on the other hand, the polysaccharide was not depolymerized until after secretion on to the membrane, then the uterine injection should have stained the shell. The experiments were negative. Of course, it has to be assumed that sufficient dye reached the uterus in one case and that the dye molecule can pass through the wall of the uterus in the other. However, when taken in conjunction with the water extraction experiment, the results suggest that depolymerized water-soluble acid mucopolysaccharide is not present in the shell.

DISCUSSION

The matrix contains some protein as shown by the protein tests and also by treatment with enzymes. Trypsin had no effect but pepsin appeared to remove the protein and liberate the acid mucopolysaccharide. Trypsin tends to attack peptide links associated with the basic amino-acids arginine and lysine, whilst pepsin attacks the peptide links of dicarboxylic amino-acids such as aspartic and glutamic acids. It is, therefore, of interest to note that Balch (1956) found both aspartic (medium reaction) and glutamic acid (strong reaction) in a hydrolysate of matrix with 'pore' protein run qualitatively on a paper chromatogram.

The various reactions with mucin stains, PAS test, and metachromatism with toluidine blue strongly suggest the presence of an acid mucopolysaccharide. The question, however, arises whether the material is chondroitin or mucoitin sulphuric acid and it is here that the experiments with hyaluronidase offer some help.

Hyaluronidase, according to Stacey (1946), first separates the protein from the polysaccharide, then rapidly depolymerizes the polysaccharide; there is then a partial or complete hydrolysis of it, which results in the liberation of acetyl glucosamine and glucuronic acid.

In experiments on the shell matrix, hyaluronidase appears to depolymerize, but not to hydrolyse the mucopolysaccharide, because it has been shown that the matrix can be repolymerized with calcium ions back to its original state. This suggests mucoitin sulphuric acid. The strong reaction to the PAS test, and the secretion of shell from the oviduct, which contains mucin-secreting cells, also tend to favour the idea of mucoitin sulphuric acid.

The combination of an acid mucopolysaccharide and a protein agrees essentially with the concept suggested by Almquist (1934), namely, that the shell matrix is a substance resembling collagen. However, it is important to recognize that the protein moiety might be quite different from collagen itself and, as already stated, the polysaccharide is probably mucoitin sulphuric acid. Support for this again comes from the qualitative chromatographic tests of Balch (1956), who showed that there was no evidence of the large amounts of hydroxyproline to be expected if the protein were collagen.

At this stage some comparison may be made with the work of Gersh and Catchpole (1949). They presented a table showing the effects of various solvents and enzymes on the reactive groups of the ground substance (glycoprotein = acid mucopolysaccharide) of mouse skin in frozen-dried material. Apart from differences in the result with trypsin, possibly explained by a difference in technique, the results with shell matrix are the same. Gersh and Catchpole consider that chemically the ground substance is relatively inert, at least as far as the carbohydrate-protein complex is concerned; a state which is consistent with the polymerized form of the material. They also believe that depolymerization of the polysaccharide complexes may result in the liberation of increasing numbers of reactive groups, some of which may combine with calcium. This depolymerization may thus play a part in the calcification of cartilage. Such a concept is obviously of great interest in the present study of the shell, where calcification is the major process in its formation. However, experiments with shells decalcified in aqueous solutions showed no indication of depolymerization. Similarly, experiments with living hens, designed to yield information similar to that provided by the injection experiments of Gersh and Catchpole, failed to reveal the presence of any water-soluble depolymerized polysaccharide, either coming from the blood or formed in the developing shell.

Recently, a more likely and better-substantiated mechanism of calcification has been put forward. Levine and others (1949) studied the disease *calcinosis universalis* and suggested that decalcification removes inorganic materials, thus making available acid groups which then stain metachromatically with toluidine blue. These acid groups cannot react with toluidine blue whilst they are blocked by elements such as calcium and thus calcified material stains orthochromatically. Later Rubin and Howard (1950) found acid mucopolysaccharide in association with calcification in bone, in calcified cartilage, and in *calcinosis universalis* and other similar conditions. They naturally suggested that the calcium-binding capacity was due to the acid mucopolysaccharide. Sobel and Burger (1954) then showed that toluidine blue, like inorganic cations, could produce a reversible inactivation of the calcification mechanism, showing that toluidine blue competes with the calcium ions for combination with something, which, in the case of ossifying cartilage, was assumed to be chondroitin sulphuric acid. An earlier paper by Sobel and Hanok (1952) had shown that the chondroitin sulphuric acid could bind much more calcium if it was coupled with a protein to give a material resembling collagen.

In the present experiments the results obtained with toluidine blue and calcium solutions were almost identical with those of Sobel, who used rachitic bones. These results therefore suggest that both the acid mucopolysaccharide and the protein components of the egg-shell matrix are vitally important to the calcification mechanism.

The mammillae appear to be basically similar to the shell matrix but to contain more protein and fat. Two of their properties, namely, the orthochromatic staining of the core with toluidine blue and a stronger reaction with the PAS test, may be explicable on the basis of a more compact organi structure and possibly a greater content of protein. However, these two properties are also the ones to be expected from a substance which is not polymerized. Thus, if calcium ions are in fact to some extent responsible fo polymerization, the mammillae may be partly laid down before calcificatio starts and a central core may remain unpolymerized.

Acid mucopolysaccharides have been found condensed with keratin in th enamel of teeth and it may be that the firm attachment of the mammillae t the membrane is brought about first by a condensation of acid mucopolysaccharide with membrane keratin.

It is possible that this suggested keratin-mucopolysaccharide complex less polymerized than true mucopolysaccharide, in which case it might giv the tests for unpolymerized material, but, because of its combination wit keratin, it might not be water-soluble. This situation could explain the difference between the present results of tests based on the work of Gersh an Catchpole and those of Gersh and Catchpole themselves. However, even this is so, the concept of calcification put forward by Sobel and Burger (1954 is the more acceptable.

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REFERENCES

Almquist, H. J., 1934. Poult. Sci., **13**, 375.
Balch, D. A., 1956. Unpublished results.
Cain, A. J., 1947. Quart. J. micr. Sci., **88**, 467.
Calverly, H. D., 1933. J. biol. Chem., **100**, 183.
Chiffelle, T. L., and Putt, F. A., 1951. Stain. Tech., **26**, 51.
Chevremont, M., and Frederic, J., 1943. Arch. Biol. Paris, **54**, 589.
Danielli, J. F., 1947. Symp. Soc. exp. Biol., **1**, 101.
Dickie, G., 1848. Ann. Mag. nat. Hist., **2**, 169.
Gersh, I., and Catchpole, H. R., 1949. Amer. J. Anat., **85**, 457.
Gomori, G., 1952. *Microscopic histochemistry*. Chicago (Univ. Press).
Haines, R. B., and Moran, T., 1940. J. Hyg., Camb., **40**, 453.
Hale, C. W., 1946. Nature, 157, 802.
Levine, M. D., Rubin, P. S., Follis, R. H., and Howard, J. E., 1949. *1st Conference metabol interrelations*, 41. New York (Macy Foundation).
Marshall, W., and Cruickshank, D. B., 1938. J. agric. Sci., **28**, 24.
McManus, J. F. A., 1948. Stain. Tech., **23**, 99.
—— and Cason, J. E., 1950. J. exp. Med., **91**, 651.
Moran, T., and Hale, H. P., 1936. J. exp. Biol., **13**, 35.
Pearse, A. G. E., 1951. Quart. J. micr. Sci., **92**, 4.
—— 1953. *Histochemistry*. London (Churchill).
Rubin, P. S., and Howard, J. E., 1950. *2nd Conference metabolic interrelations*, 155. New York (Macy Foundation).
Sobel, A. E., and Burger, M., 1954. Proc. Soc. exp. Biol. N.Y., **87**, 7.
—— and Hanok, A., 1952. J. biol. Chem., **197**, 669.
Stacey, M., 1946. *Advances in carbohydrate chemistry*, vol. 2, 162. New York (Academ Press).
Steedman, H. F., 1950. Quart. J. micr. Sci., **91**, 477.
Stewart, G. F., 1935. Poult. Sci., **14**, 24.
Tyler, C., 1956. J. Sci. Fd. Agric., **7**, 483.
—— and Geake, F., 1953. Ibid., **6**, 261.

A Histochemical Study of the Connective Tissue-Fibres in the Leech, *Glossiphonia complanata*

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With plate (fig. 2)

SUMMARY

The fibres and the connective tissue ground-substance of *Glossiphonia complanata* have been studied morphologically and histochemically. The fibres, which run in the connective tissue-substance between the organs of the body, are cytoplasmic extensions of bipolar or multipolar cells. The average diameter of the fibres is between 1 and 2 μ, and a single fibre may exceed 100 μ in length. Each fibre is differentiated into a cortex and a medulla. The cortex contains arginine, and some acid and neutral mucopolysaccharides; this suggests that it is collagenous. The medulla is an extension of the protoplasm of the cell-body.

The cell-body of the fibre has a basiphil cytoplasm which contains arginine, acid and neutral mucopolysaccharides, and much ribonucleic acid. Spherical lipochondria up to 4 μ in diameter are present in the cytoplasm, especially near the nucleus; they contain chiefly phospholipid. Threadlike mitochondria are present in the cytoplasm of this cell.

The connective tissue ground-substance is present as a matrix between the cells and organs of the body. Histochemical tests showed that it contains small amounts of arginine, tyrosine, neutral and acid mucopolysaccharides. The refractive index of the ground-substance is about 1·35.

THE strength and resilience of the connective tissues of leeches is well known; in some species, e.g. *Glossiphonia complanata*, it prevents easy isolation of the organs and cells of the body. Much of this strength is due to the presence in the ground connective tissue-substance of interlacing fibres; these were noted and figured by Bourne (1884), Scriban (1910, 1923), Abeloos (1925), and Scriban and Autrum (1934) among others. No histochemical study of these fibres appears to have been published, though deductions as to their nature have been made on the basis of their general staining characteristics. It is the purpose of this paper to give the results of histochemical studies of these fibres, and on the substance which forms the intercellular matrix.

MATERIAL AND METHODS

Material was obtained from the fresh-water rhynchobdellid leech, *G. complanata*. Much of the work was carried out on fixed material, studied in sections, but fresh tissue was also examined with the phase contrast microscope and with the Baker interference microscope. Details of the histochemical tests are given in the appendix.

The connective tissue-fibres

General. In any transverse section of *G. complanata* stained with suitable techniques, a mass of fibres can be seen in the connective tissue between the

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muscles and other organs of the body; these fibres were considered by Scriba and Autrum (1934) to arise from embryonic connective tissue-cells by modification involving extension of processes from the cell-body. They stat that the majority of these fibres originate from bipolar cells, though som especially in the region surrounding the ganglion chain, have multipola stellate cells as their source.

The fibres are embedded in the ground-substance of the connective tissu and it is stated by these authors that their axes generally lie in the long axi

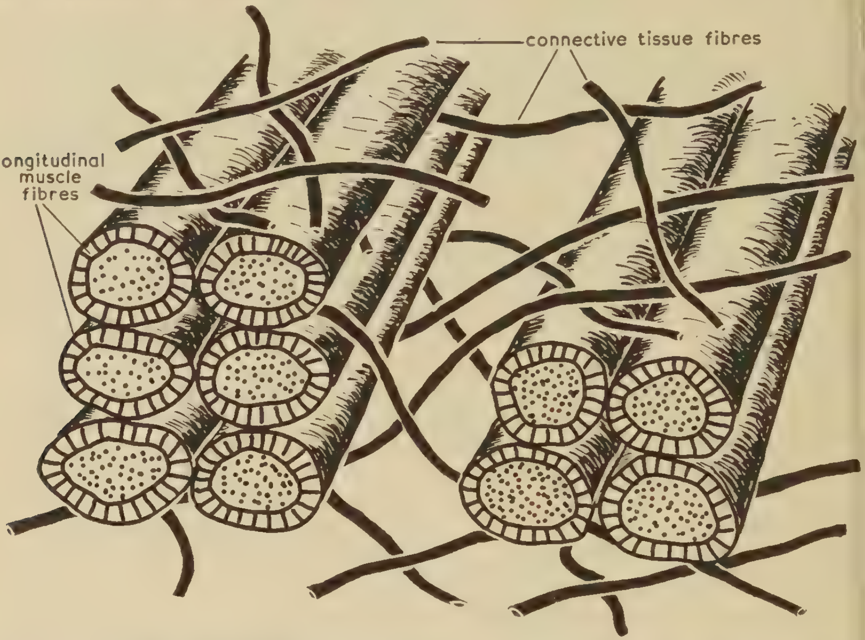

Fig. 1. A schematic representation of the arrangement of the connective tissue-fibres and th longitudinal muscles. Both are embedded in the ground-substance of the connective tissu which is not represented in the diagram.

of the body. From my study of sections cut transversely and also longitudinally, it appears that the general tendency is for the fibres to run obliquely along the body, so forming an interlacing system. This is represented very diagrammatically in fig. 1, where the fibre paths between muscle-bands are shown. In the figure the course of the fibres is represented for the sake o clarity as being much more regular than is the case (many of the fibres have been omitted for the same reason). In the deeper regions of the body, e.g around the lateral coelomic sinuses, the fibres appear to be much more random in their arrangement, but there may be special concentrations aroun certain organs such as the testis sacs.

It has been noticed in the course of the present study that there is a tendency for oblique fibres running between the longitudinal muscle-blocks to cross over, so that fibres which had been running, for instance, below the

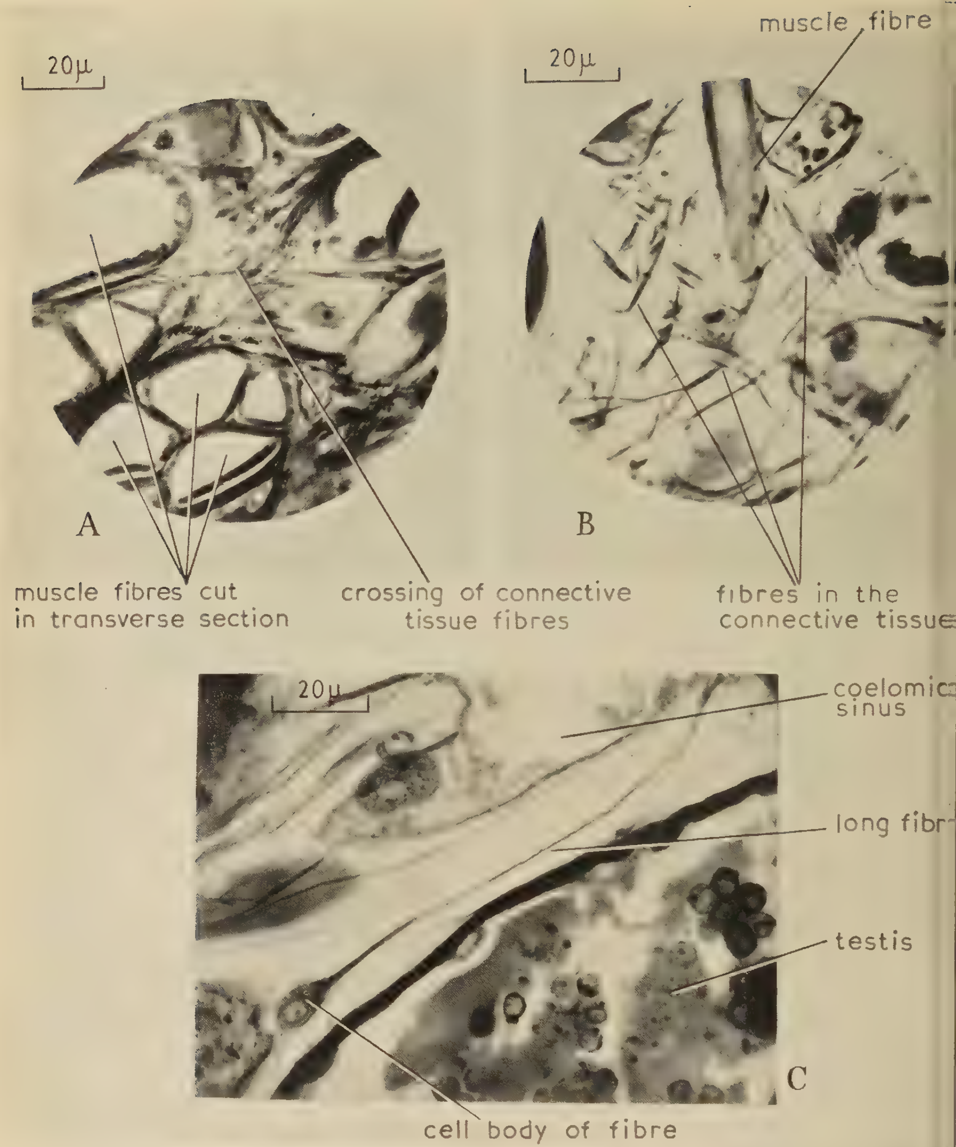
20μ
20μ
muscle fibre
A
B
muscle fibres cut in transverse section
crossing of connective tissue fibres
fibres in the connective tissue
20μ
coelomic sinus
long fibr
testis
C
cell body of fibre

FIG. 2

S. BRADBURY

orso-median muscle-block may eventually be found beneath the dorso-ateral muscles, and so on. This is seen in fig. 1, and also in fig. 2, A, which hows such a crossing in the lateral coelomic sinus region of the body; it may e that this is a device for ensuring the maximum possible strength consisent with the greatest degree of body flexibility, though the actual crossing ver of fibres would not of itself give rise to increased mechanical strength. This would only be so if the fibres were attached to some part of the body tructure, either at their ends or along their length, or if the fibres were ttached to each other, or, alternatively, if they were totally bound up along he whole of their length with a surrounding matrix.

In *Glossiphonia*, the method of fibre termination has not been observed, o that no conclusion can be drawn about the first of these possibilities, but it s possible to say that no attachments along the fibre length have been seen. This point contrasts with the findings of Cowey (1952) for the fibres in the asement membrane system of the Nemertine *Amphiporus*. From his results, t is clear that in this animal the fibres (which seem to be collagenous) do ctually make contact with other fibres. A geodetic system is thus formed, nd this ensures that there is no slipping of the fibres relatively to each other n contraction or extension of the animal.

It seems likely, however, that in the case of *Glossiphonia* the third possibility olds good. The difficulty of extracting whole cells and fibres from the body oints to the existence of some strong ground matrix, as do all the histochemial results outlined later in the paper. If the fibres are assumed to be in ntimate contact with this ground-substance throughout their length, then his arrangement would be expected to confer great strength and flexibility n the animal.

The fibres do not appear to branch, although it is difficult to see how they erminate, on account of their small size and because it is rare for the whole ength of any one fibre to be included in the plane of a section. This compliates direct measurement of fibre length, but by the use of thick celloidin ections it has been possible to follow individual fibres for distances of over 00 μ along their length (fig. 2, C). Isolated fibres have been obtained from naterial which has been macerated for several days, but in no experiment did hey exceed 100 μ in length and they were usually much shorter. Measurenents of fibre diameter, however, are relatively easy and the values obtained ave an average diameter of between 1 and 2 μ.

The connective tissue-fibres are not homogeneous, but are clearly differeniated into a medulla, which is an extension of the protoplasm of the cell-body long the axis of the fibre, and a cortex. This distinction is clearly shown in

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FIG. 2 (plate). The connective tissue-fibres of *Glossiphonia complanata*. All impregnated ith osmium.

A, transverse section in the region of the lateral coelomic sinus. Note the crossing of fibres etween two groups of muscle-fibres.

B, fibres in the deep-seated connective tissue. Note that they run singly and not in bundles.

C, a single fibre in the connective tissue, near the testis sac. The origin from a single cell n be clearly seen.

fig. 3, taken from Scriban and Autrum (1934). The fibres represented i the figure are from *Pontobdella muricata* and *Hirudo medicinalis*, but the

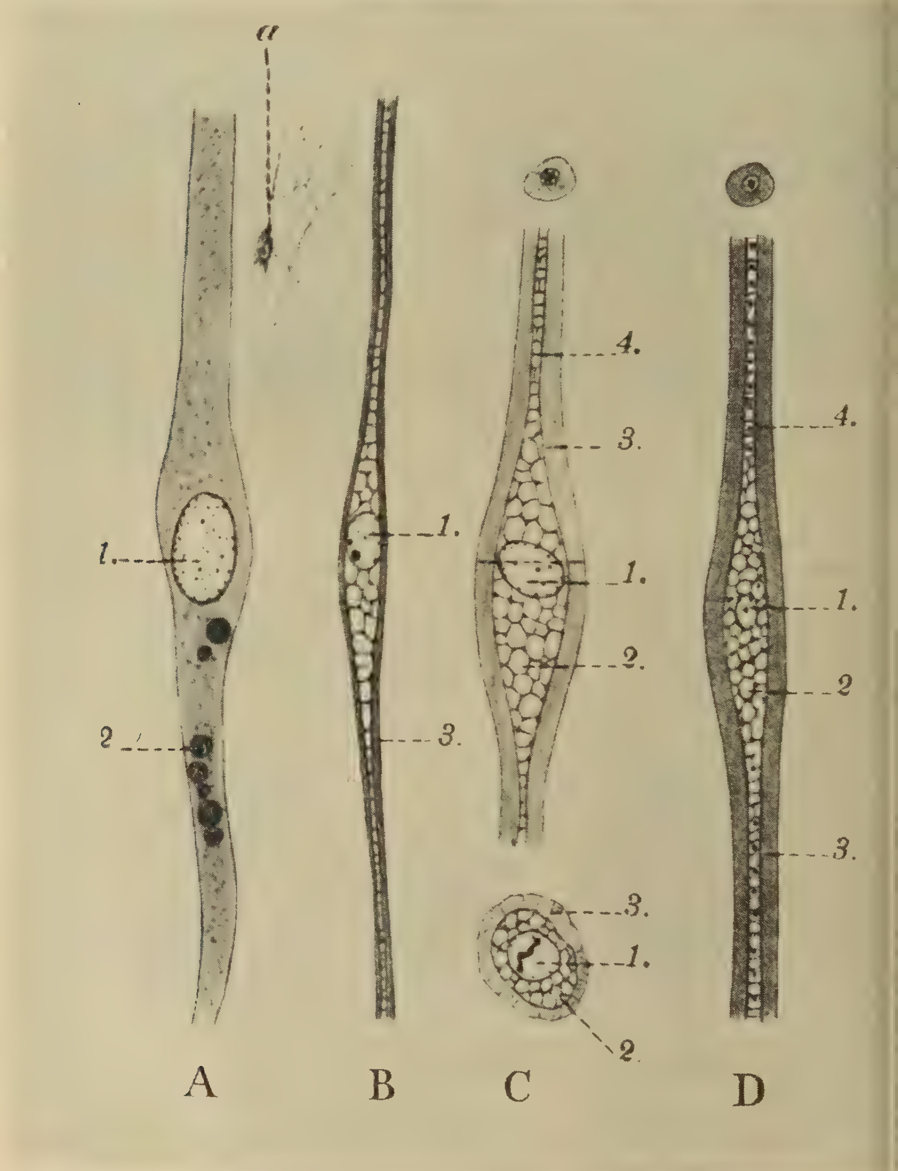

FIG. 3. Connective tissue-fibres of other leeches. A, B, and C from *Pontobdella muricata*, from *Hirudo medicinalis*. Note the cortex and medulla, also lipochondria in the cytoplasm c fibre A. Figure taken from Scriban and Autrum (1934).

resemble very closely those found in *Glossiphonia*. It is noticeable that, i the figure, the cortex of the fibre is very pronounced and is represented a extending unchanged over the body of the cell, whereas in *Glossiphonia*, though the fibre cortex does extend over the cell-body, it is not marked in this region, but is represented here by a thin layer only.

The cytoplasm of the cell-body extends down the fibre axis, and is shown in the figure as possessing a marked alveolar nature. In the present study this appearance was noticed only in material which had been fixed in a coagulant fixative; in studies of unfixed tissues, or of tissues fixed in formaldehyde, the alveoli were not at all obvious; so it may be that they do not represent structure present in the living cell, but are fixation artifacts. The collagenous nature of the fibre cortex was inferred by most of the previous workers from the noted swelling of the fibre when treated with acetic acid, and from the general staining characteristics of the fibre. The fibres were found to give all the colour reactions appropriate to collagen (see appendix). Although these staining reactions afford very strong presumptive evidence that the substance of the fibres is, in fact, collagen, they do not constitute histochemical proof; so in order to try to establish the identity of this substance beyond doubt, a series of more precise histochemical tests was undertaken.

Histochemistry

Lipids. The cortex of the fibres did not give a positive reaction to any of the standard tests for lipids; the axial protoplasm, however, sometimes contained small globules which resembled the lipochondria of the cell-body. It seems that the cortical material of the fibres contains no free lipids detectable with the histochemical tests in current use, and that the lipid which occurs in the medulla of the fibres is restricted to small lipochondria concentrated near the cell-body.

Amino-acids and proteins. Only three methods were used, namely, the Hg / nitrite test (Baker, 1956) for phenols, especially tyrosine, the same author's modification of the Sakaguchi test for arginine and other guanidine derivatives, and the 'coupled tetrazonium' reaction (Danielli, 1947; Pearse, 1954).

With the Hg / nitrite test, negative results were obtained, the fibres not colouring at all, so that it seems probable that tyrosine does not form any appreciable part of these structures. Sakaguchi's reaction, on the other hand, gave a fairly strong coloration of the fibres, although the colour was not so intense as that given by the nuclei in the section. This makes it seem very probable that arginine enters into the composition of these fibres, but it was not possible to decide to what extent the cortex or medulla of the fibre was responsible for this particular reaction. In view of the observation that the cell-body was only very feebly coloured in this test (implying a low concentration of arginine), it seems probable that the same would be true of the protoplasm forming the medulla of the fibre, so the intense reaction may provisionally be attributed to the cortical portion of the fibre.

The 'coupled tetrazonium' reaction was rather weakly positive for the fibres, but if the sections were treated with mild heat and benzoylation according to the directions given by Pearse (1954), before doing the reaction, then the fibres coloured much more strongly. In his book Pearse shows that thi result is characteristic of collagen, among other substances, the applicatio of heat being presumed to cause some physical change by which the reactiv groups of tryptophane and certain other amino-acids are prevented fro combining with benzoyl chloride but remain active to diazonium salts.

Though the amino-acid content of collagen has been known for a lon time, very exact analyses have not been available until much more recentl (e.g. Bowes and Kenten, 1949; Randall, 1953); as a result of such work it i known that there is much more arginine than tyrosine in collagen. Thi accords very well with the observed histochemical reactions, the Sakaguch test for arginine being strongly positive, whereas the test for tyrosine is entirel negative. On the basis of the histochemical tests for amino-acids, therefore it seems reasonable to suppose that some part of the fibre is composed o collagen.

Carbohydrates. When the PAS reaction was applied to the material, th results were rather indecisive, the fibres colouring only very slightly pink As a control, sections were subjected to the PAS reaction after incubation i saliva; some of the positive reaction was abolished by this treatment, e.g. i the muscle-fibres, but the connective tissue-fibres were still faintly coloure by the Schiff's reagent. It may be concluded that there is a small amount o carbohydrate present in association with the fibres, but the reaction was s feeble that it was impossible to say whether it was concentrated in the corte of the fibre or, as proposed for collagen fibres in other material, actually on the surface of the fibre.

In order to see whether the positive reaction could be due to the presence of an acid mucopolysaccharide, sections were stained in toluidine blue an examined for the occurrence of metachromasy. It was found that the fibres showed a very slight degree of metachromasy, which was much more apparent in thick sections. Again there was some doubt as to whether the reactio was more intense in the actual fibres, or whether the reacting substance wa located chiefly in the intercellular material which surrounds them. As it was possible that some of the polysaccharide existed in the form of a neutra mucopolysaccharide, the technique recommended by Lison (1953), involvin treatment of the sections with concentrated sulphuric acid for a very shor time before staining with toluidine blue, was used to check this point. Afte this technique, it was found that the intercellular material, as well as th fibres, became intensely metachromatic, so that it seems extremely probabl that much of the carbohydrate is actually present in the form of a neutra mucopolysaccharide. If this is the case, treatment with chromic acid befor staining should cause the appearance of metachromasy (Lison, 1953) bu with this particular tissue this experiment was unsuccessful. It is concluded however, on the basis of the other results, that there is a neutral mucopolysaccharide both in the connective tissue matrix, and either in or on the surfac of the fibres.

As the experiments with toluidine blue suggested the presence of an aci ucopolysaccharide in small amounts, it was decided to measure the basiphilia f the tissues by means of the methylene blue extinction method (Pischinger, 927; Dempsey and Singer, 1946; Pearse, 1954), as the capacity to bind ethylene blue at a very low pH is usually indicative of sulphate groups, i.e. cid mucopolysaccharides, or of nucleic acids. In this experiment, the value bserved for the fibres was between pH 4·6 and 5, so that this gave no evidence or the existence of an acid mucopolysaccharide. Although the methylene lue extinction method cannot be considered as giving an accurate measure f the isoelectric point of any tissue component, it serves to give some indi-ation of its value, and when taken together with the other evidence may urnish useful information; it is, therefore, interesting to note that the figure f pH 4·6 to 5 is very close to the isoelectric point of collagen as quoted by earse (1954) in his table 8.

Miscellaneous tests. As the fibres are probably in active growth for a con-iderable period of the life of the animal, it was thought possible that there ight be an accumulation of nucleic acid in the cytoplasm of the fibre. When he pyronin / methyl green technique of Jordan and Baker (1955) was tried, t was found that although a strongly positive reaction was given by the ctual cell-body, there was no result for the fibre. This was also true for the eulgen test for deoxyribonucleic acid, which was shown to give a positive esult only in the nucleus of the fibre-cell.

In view of work by Moog and Wenger (1952) and Goreau (1956), drawing ttention to the occurrence of mucopolysaccharide at sites of high phosphatase ctivity, it was thought possible that a similar condition might occur in *lossiphonia*. Tests were tried for both acid and alkaline phosphatases, but o positive reaction could be found in the connective tissues; so it seems that n this animal no relationship exists between the mucopolysaccharide content f the connective tissues and the occurrence of phosphatase.

Though the method of Weigert for staining elastin is not histochemically pecific, it gives a good indication as to the presence or absence of elastic bres. In this leech, the results of such a test are negative, a finding which is upported by the histochemical results already considered. Elastin contains ttle arginine, but more tyrosine, and has an isoelectric point much closer to eutrality than that of collagen, so that if elastin occurred in these fibres to ny extent, the Sakaguchi reaction would not be expected to be strong, but he Hg / nitrite reaction for tyrosine would be expected to give a positive esult. It thus seems reasonable to assume that the fibres of *Glossiphonia* do ot contain any elastin; they do, however, give histochemical and other eactions which are entirely consistent with the hypothesis that they are ainly composed of collagen and probably contain, in addition, small quan-ties of both acid and neutral mucopolysaccharides.

he fibre cell-body

General. The structure of the cell-body of a connective tissue-fibre is hown in diagrammatic form in fig. 4. The majority of these cells are bipolar, though as mentioned by Scriban and Autrum (1934), multipolar ones occu in the connective tissue around the nerve-cord. The actual cell-body between 15 and 20 μ long, and between 7 and 10 μ in diameter; its ends ar drawn out into the long processes which form the connective tissue-fibre The cortex of the fibre continues over the body of the cell, though it is ver thin in this region. The cytoplasm of the cell is basiphil and stains easily wit dye lakes, such as haemalum; in most fixed material the cytoplasm appear rather granular, but in no preparation was any structure observed whic resembled the 'surround' noticed in the adipose cells of the same species.

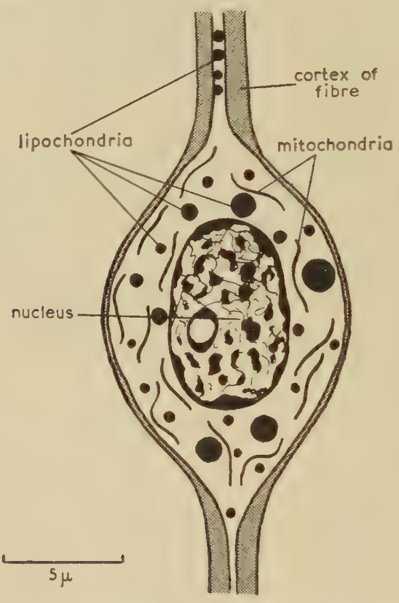

FIG. 4. Diagram of a generalized fibre-cell of *Glossiphonia complanata*.

The nucleus of the fibre-cell is oval, usually measuring 7 or 8 μ along it length; there is usually a very well-developed nucleolus. Only two types o cytoplasmic inclusion, mitochondria and lipochondria, have been noticed i these cells. The mitochondria appear as straight or slightly curved rods about 3 μ in length and 0·5 μ in diameter, scattered throughout the cytoplasm of the cell, with no apparent concentration in any particular region. Th lipochondria are spherical, with diameters from 1 μ up to about 4 μ; the occur throughout the cytoplasm, perhaps with some tendency to aggregat near the nucleus. Similar bodies also occur in the medulla of the fibrou processes of this cell.

Histochemistry. The histochemical tests carried out on these cells are liste in the appendix. The tests show two significant features; the cells posses much arginine, as revealed by the Sakaguchi test, and they show a pronounce degree of basiphilia. Some indication of this is given by the methylene blu extinction value for the cells, which is about pH 2·6, a figure very differe from the value for the fibres. The pyronin / methyl green technique showed that this cytoplasm coloured strongly with pyronin, so it seemed probable that the basiphilia was due to the presence of ribonucleic acid. It has been demonstrated (Bradbury, 1956) that when slides are incubated in a bath of treated saliva, the basiphilia due to ribonucleic acid is abolished; in this cell, the treatment with saliva entirely prevented coloration of the cytoplasm by the pyronin, so it is concluded that the basiphilia is due to the presence of ribonucleic acid.

The usual tests for lipids were tried, but positive reactions were only obtained from the lipochondria. These were very strongly coloured by Baker's acid haematein technique, as also were the mitochondria; they did not colour blue after pyridine extraction, so both inclusions appear to contain large quantities of phospholipid. As two types of fatty inclusions, one composed of neutral fat, and the other of phospholipid, were found in the adipose cells of *Glossiphonia*, it was decided to check whether a similar condition existed in this cell. When the acid haematein test is followed by Sudan IV, all lipids which are not coloured blue by the acid haematein will be revealed in red. In this cell, no fat drops coloured with the Sudan dye, so that it seems likely that only one series of fatty inclusions is present.

It has been noted in other material, e.g. the sympathetic neurone of the mammal (Casselman and Baker, 1955), cerebrosides are very often associated with the lipochondria. These compounds are not affected by fixation in cold acetone, but they are removed from the material if the acetone is kept near its boiling-point in a simple reflux apparatus. Any difference which is apparent between sections from material treated in these two ways and then coloured with Sudan black may be attributed to the presence of a cerebroside. In *Glossiphonia* it was found that no lipids could be detected after fixation in either hot or cold acetone, and it is therefore probable that in this cell the phospholipid is not in association with a cerebroside.

The ground-substance

The existence of some form of matrix between the cells and organs of the vertebrate body has been recognized for a considerable time, though some authors, e.g. Nageotte (1922), have questioned this view. Nageotte and his followers deny the existence of an amorphous substance, considering that the spaces between the components of the body are empty except for tissue-fluids. Bensley (1934) has gone into the evidence for the existence of an intercellular ground-substance in vertebrates, and shows that there are very strong grounds for assuming its existence. Among other experiments, she measured its refractive index, demonstrated that it has an affinity for metallic ions, and found that it can be differentially stained with toluidine blue.

The ground-substance of the connective tissue of *Glossiphonia* appears perfectly homogeneous in unstained sections examined by phase contrast microscopy, but it may easily be recognized in sections by its uniform coloration with standard histological techniques such as Masson or picro-indigo-carmine. The ground-substance may also be revealed by histochemical tes such as the PAS reaction or by its differential staining with toluidine blu As Bensley pointed out for the ground-substance of vertebrate connectiv tissue, there is a marked affinity for metallic ions and this property may b utilized to show very clearly the extent of the ground-substance in *Gloss* *phonia*. With osmium impregnation, this substance appears coloured a un form brown and is seen to surround all the organs of the body; in it ar

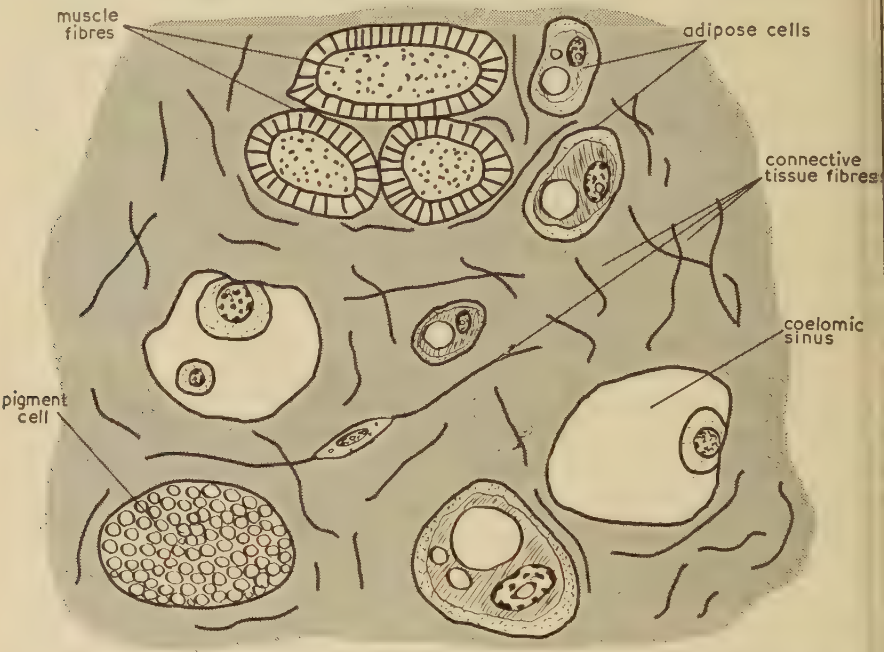

FIG. 5. A diagrammatic transverse section of part of the body of *Glossiphonia complanata* in the region of the lateral coelomic sinus. The extent of the ground-substance of the connective tissue is shown in stipple.

embedded the muscles, connective tissue-fibres, pigment cells, adipose cells, the coelomic sinus system, and all the other structures of the body. Fig. 5 represents diagrammatically part of the animal seen in transverse section with the area occupied by the ground-substance indicated in stipple.

Histochemistry of the ground substance

It is seen from the appendix that all the tests for lipids are negative, as are those for enzymes and for nucleic acids.

The 'coupled tetrazonium' reaction was weakly positive, indicating the presence of a small amount of protein; weak positive results were also given by Baker's Hg / nitrite test and Sakaguchi's test, so that some arginine and tyrosine are present, though neither seems to be very abundant.

The PAS reaction is weakly positive, with no decrease in intensity when the sections are treated with saliva before the test. This seems to rule out the possibility of a reaction due to glycogen. It suggests instead the presence of either an acid or a neutral mucopolysaccharide. When sections are stained with toluidine blue, a very weak metachromasy of the ground substance is noticed; treatment with concentrated sulphuric acid before staining with toluidine blue gives a strong metachromasy, as in the case of the fibres: this evidence supports the view that a neutral mucopolysaccharide is present. There was no increase in metachromasy nor in the intensity of the PAS reaction after treatment with chromic acid. Methylene blue extinction occurred between pH 5 and 6; this result does not give any significant information about the composition of the ground substance.

The optical path difference due to the intercellular substance (after fixation) was measured with the Baker interference microscope, and the refractive index calculated. A small fragment of a section of the fixed tissue, consisting mainly of intercellular substance, was placed in the microscopic field so that it occupied only a small part of the field of view; the area unoccupied by the section served as a reference area. The measurements of the optical path difference were obtained by rotating the analyser so that first the reference area and then the intercellular tissue of the section were as dark as possible when monochromatic green light was used. When these conditions were fulfilled, the readings of the goniometer of the analyser were noted. It can be shown theoretically that the optical path difference represents twice the difference between these two readings, hence the refractive index of the tissue may be calculated by using the formula

$$u = \frac{\theta\lambda}{360\,t} + n,$$

where u = the refractive index of the object,
θ = the optical path difference expressed in degrees,
t = the thickness of the object in μ (in this case the thickness of the section),
n = the refractive index of the mounting medium,
and λ = the wavelength of the light used.

Measurements of the refractive index calculated from readings taken with the object mounted in water and from other readings with the object in glycerine were very similar; the mean of five measurements was 1·35, a figure very close to that obtained by Bensley (1934) for the connective tissue ground-substance of vertebrates. Her method was to immerse the tissue in different media of known refractive indices and note when the tissue became difficult to distinguish from the background. The refractive index of the fluid in which this condition was fulfilled was taken as a close approximation to that of the tissue. The value measured for *Glossiphonia* is rather low, which suggests that the ground-substance does not contain much solid matter.

In an attempt to confirm the histochemical results on the carbohydrates by an alternative method, paper chromatography was used, though when used as described below it has the great drawback that the source of the extract is not precisely known. The experiments to be described show that the extracted substances are PAS positive and metachromatic, and show increase of metachromasy on oxidation. Histochemical study of sections showed that if glycogen is excluded, the only substances in the body which fulfilled all these conditions were in the cuticle, in the connective tissue-fibres, in the connective tissue ground-substance, or in very small amounts in the adipose cells.

The possibility of glycogen being responsible was excluded by using starved animals. Furthermore, as the amount of material extracted was quite considerable, the adipose cells and the cuticle may be disregarded; and so, by exclusion, it seems that the substances subjected to paper chromatography must be derived from the fibres, or more probably from the ground-substance.

The extraction of the material was carried out by homogenizing two leeches in 1 ml of 10% aqueous calcium chloride solution, a solvent which was used by Meyer and Smith (1937) in their studies of the acid mucopolysaccharide, chondroitin sulphate. The aqueous extract was allowed to stand overnight at a temperature of 4° C before centrifuging in order to remove the cell debris. Spots of the supernatant liquid were placed on Whatman's no. 1 paper, with samples of commercial chondroitin sulphate and heparin as comparison substances. A mixture of 0·25% propanol in M/15 phosphate buffer at pH 6·4 was used as a solvent, as recommended by Kerby (1953) for the separation of chondroitin sulphate and heparin. After the chromatogram had been run, the spots were developed either by spraying the paper with an 0·25% aqueous solution of toluidine blue, by performing the PAS reaction on the paper, or by oxidizing the substances with periodate before spraying with toluidine blue.

In the chromatograms developed with toluidine blue alone, the chondroitin sulphate and the heparin gave well-defined metachromatic spots, but there was no spot from substances present in the extract. When the paper was dried, however, a faint metachromatic spot appeared in a position which suggested that it was due to some substance present in the leech extract. No explanation can be put forward to account for the fact that this substance only showed metachromasy when the paper was dry; possibly the substance responsible may only have been present in extremely small amounts, or it may be the result of considerable structural change induced in the molecule of the substance by drying. The PAS reaction showed a spot, due to some substance in the extract, which coincided in position with that shown by the toluidine blue; in both cases, the spots did not coincide with those due to the heparin or the chondroitin sulphate. After oxidation by means of periodate, the spot due to the extract was much more spread out, much larger, and appeared intensely metachromatic. This may be a result of the oxidation of a neutral mucopolysaccharide by the periodate, so giving a product which is metachromatic.

These experiments seem to confirm the presence of small amounts of an acidic, and also larger amounts of a neutral mucopolysaccharide in an extract from *Glossiphonia*. The location of these substances cannot be deduced from chromatography, but the histochemical work shows that both probably occur together in the ground-substance of the connective tissue and the fibres.

DISCUSSION

It seems clear from a consideration of the results given in this paper that the fibres found in the connective tissue of *Glossiphonia complanata* differ very considerably in structure and arrangement from those of vertebrates. The fibres are sharply marked off into a cortex and a medulla; are clearly long extensions of the actual fibre-forming cells; and retain a connexion with the cells which secrete them. These are all points of difference from the vertebrates. In addition, the fibres of *Glossiphonia* never seem to associate into large bundles, so commonly found in vertebrate animals, but remain single and interlace among each other in the ground-substance. There is a resemblance to vertebrates in that the fibres almost certainly contain collagen, together with mucopolysaccharides. Several problems still remain, however, in particular the questions of fibrogenesis, of the cellular state of the fibres, and the origin of the ground-substance.

Even in the vertebrates, the problem of fibrogenesis is still not solved; in particular there is still doubt whether the fibres are secreted intra- or extracellularly. Recent work published by Fitton Jackson (1955) suggests that even if the fibres are formed outside the cell, the actual process of formation is closely linked to events within the cell. In chick fibroblasts, granules have been observed in the protoplasm of the cell. These granules have been shown to consist of carbohydrates and some protein, and to possess alkaline phosphatase. It is suggested that these granules may be the precursors of the whole or part of the ground-substance, or possibly of the collagenous fibre itself. Extrusion of the granules into the ground-substance has not been noticed by this author but has been claimed by other workers, e.g. Stearns (1940). No such granules have been observed in the fibre cell-bodies of *Glossiphonia*, but a similarity does exist between the granules seen in the chick and the ground cytoplasm of the fibre-cells in *Glossiphonia* with respect to their chemical composition, so far as it can be determined. A conspicuous difference is that no phosphatase activity has been detected in the leech tissue.

With the methods at present available, it does not seem possible to determine whether the collagen forming the cortex of the fibre is actually deposited on the inner surface of the cell-membrane, or whether it is laid down on the outer side of the membrane, when the fibre might be considered to be extracellular.

Histochemical study of developing embryos of this leech could give interesting results, and might perhaps throw some light on the problem of whether the fibre consists of some other substance before the collagen is deposited.

The ground-substance of the connective tissue shows similarities i chemical composition to that of vertebrates. Measurement of the refractiv index suggests that the solid content is rather low. This may indicate tha this ground-substance exists as a semi-fluid gel. Histochemical studies d not help much in deciding the origin of the ground-substance. It has bee thought to arise in several ways, e.g. Bourne (1884) considered that it resulte from 'ectoplastic modification of the corpuscles lying within it'. A more recen view is that the ground-substance is the result of secretion by the connectiv tissue-cells. As there are chemical similarities between the composition of th ground-substance and the fibre-producing cells, this does not seem entirel unreasonable. Future work with histochemical methods on the developin tissue may help us to understand this process more clearly.

I have pleasure in acknowledging my debt to Dr. J. R. Baker for supervising this work and for stimulating discussions. I also wish to thank Professor A. C Hardy, F.R.S., in whose department the work was done, and Dr. P. C. J Brunet for teaching me some essentials of chromatographic technique.

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REFERENCES

Abeloos, M., 1925. Bull. Biol. Fr. et Belg., **59**, 436.
Baker, J. R., 1944. Quart. J. micr. Sci., **85**, 1.
—— 1946. Quart. J. micr. Sci., **87**, 441.
—— 1947. Ibid., **88**, 115.
—— 1949. Ibid., **90**, 293.
—— 1956. Ibid., **97**, 161.
Bensley, S. H., 1934. Anat. Rec., **60**, 93.
Bourne, A. G., 1884. Quart. J. micr. Sci., **24**, 419.
Bowes, J. H., and Kenten, R. H., 1949. Biochem. J. **45**, 281.
Bradbury, S., 1956. Quart. J. micr. Sci., **97**, 323.
Calleja, C., 1898. Z. wiss. Mikr., **15**, 322.
Casselman, W. G. B., and Baker, J. R., 1955. Quart. J. micr. Sci., **96**, 49.
Cowey, J. B., 1952. Ibid., **93**, 1.
Danielli, J. F., 1947. Symp. Soc. exp. Biol., **1**, 101.
Dempsey, E. W., and Singer, M., 1946. Endocrinology, **38**, 270.
Feulgen, R., and Rossenbeck, H., 1924. Z. phys. Chem., **135**, 203.
Fitton Jackson, S., 1955. Nature, **175**, 39.
Gomori, G., 1952. *Microscopic histochemistry*. Chicago (University Press).
Goreau, T., 1956. Nature, **177**, 1029.
Jordan, B. M., and Baker, J. R., 1955. Quart. J. micr. Sci., **96**, 177.
Kerby, G. P., 1953. Proc. Soc. exp. Biol. Med., **83**, 263.
Lison, L., 1953. *Histochimie et cytochimie animales*. Paris (Gauthier-Villars).
Mallory, F. B., 1900. J. exp. Med., **5**, 15.
—— 1938. *Pathological technique*. Saunders (Philadelphia).
Mann, G., 1902. *Physiological histology*. Oxford (Clarendon Press).
Meyer, K., and Smith, E. M., 1937. J. biol. Chem., **119**, 507.
Moog, F., and Wenger, E. L., 1952. Am. J. Anat., **90**, 339.
Nageotte, J., 1922. C. r. Soc. Biol. Paris, **87**, 147.
Pantin, C. F. A., 1948. *Microscopical technique*. Cambridge (University Press).

Pischinger, A., 1927. Pflug. Arch. ges. Physiol., **217**, 205.
Pearse, A. G. E., 1954. *Histochemistry, theoretical and applied.* London (Churchill).
Randall, J. T., 1953. *Nature and structure of collagen.* London (Butterworth).
Schafer, E. S., 1949. *Essentials of histology.* London (Longmans).
Scriban, J. A., 1910. Ann. Sci. Univ. Jassy, **6**, 147.
—— 1923. C. r. Soc. Biol. Paris, **88**, 935.
—— and Autrum, H., 1934. 'Hirudinea' in Kükenthal and Krumbach's *Handbuch der Zoologie.* Berlin (De Gruyter).
Stearns, M. L., 1940. Amer. J. Anat., **67**, 55.
Weigert, C., 1898. Centralbl. f. allg. Path., **9**, 289.

APPENDIX

A summary of the histochemistry of the connective tissue-fibres and of the connective tissue ground-substance of Glossiphonia complanata

Practical notes					Results		
Test or technique	*Fixation*	*Embedding medium*	*Thickness of section in μ*	*Reference*	*Connective tissue-fibre*	*Connective tissue-fibre cell-body*	*Connective tissue ground-substance*
Mallory's trichrome	Z, C, F.S	P	8	Mallory, 1900	blue	red	pale blue
Masson	Z	P	8	Pantin, 1948	green	red	green
Picro-indigo carmine	Z	P	8	Calleja, 1898	blue	orange red	pale blue
Mallory's collagen stain	Z	P	8	Mallory, 1938	blue	yellow	pale blue
Mann's methyl blue-eosin	Z C	P	8	Mann, 1902	blue	orange	blue
Gram	Z	P	8	Mallory, 1938	+reddish	—	weak+
Acid fuchsin	Z C	P	8		+	O	O
Orcein / Van Gieson	Z	P	8	Schafer, 1949	pink	yellow	O
Weigert	C	P	8	Weigert, 1898	O	O	O
Sudan black	FS+PC FCa+PC	G	10	Baker, 1944, 1949	O	O ++ small lipochondria	O O
Acid haematein	FCa+PC	G	10	Baker, 1946	O	++lipochondria ++mitochondria	O
Acid haematein: pyridine extraction	WB+PE	G	10	Baker, 1946	O	O	O
Leibermann	FS	G	10	Lison, 1953	O	O	O
Windaus	FS	G	10	Lison, 1953	O	O	O
Sudan black after cold acetone	AC	G	10	Pearse, 1954	O	O	O
Sudan black after hot acetone	AC	G	10	Pearse, 1954	O	O	O
Hg / nitrite	FS	C	15	Baker, 1956	O	O	+
Sakaguchi	Z	P	8	Baker, 1947	++	+++	O
Coupled tetrazonium	FS	P	8	Pearse, 1954	weak+	weak+	O

CT+heat+benzoylation	FS	P	8	Pearse, 1954	++	weak+	O
PAS	FS Z	P	8	Pearse, 1954	weak+	O	very weak +
PAS after saliva	FS Z	P	8	Pearse, 1954	weak+	O	weak+
Toluidine blue for metachromasy	FS Z	P	8	Baker, unpublished	+γ	?+γ	+γ
Toluidine blue after chromic acid	FS Z	P	8	Lison, 1953	O	O	O
Toluidine blue after sulphuric acid	FS Z	P	8	Lison, 1953	+++γ	+++γ	+++γ
Methylene blue extinction	FS	P	8	Pearse, 1954	pH 4·6–5	below 2·6	pH 5–6
Pyronin / methyl green	Z3H	P	8	Jordan and Baker, 1955	O	pyronin+++	O
Pyronin / methyl green after saliva	Z3H	P	8	Bradbury, 1956	O	O	O
Feulgen	Z	P	8	Feulgen and Rossenbeck, 1924	O	nucleus +++	O
Feulgen: no hydrolysis	Z	P	8	Feulgen and Rossenbeck, 1924	O	O	O
Gomori for acid phosphatase	ALC / AC	P	8	Gomori, 1952	O	O	O
Gomori for alkaline phosphatase	ALC / AC	P	8	Gomori, 1952	O	O	O
Osmic impregnation	MF+ IMPR 12D	P C	8 20		++	Lipochondria+++ O	+

Key: AC = fixation in absolute acetone; ALC / AC = absolute alcohol / acetone mixture; C = celloidin; F = neutral formaldehyde; FCa = formaldehyde calcium; FS = formaldehyde saline; G = gelatine; +IMPR 12D = osmic impregnation for 12 days; MF = Mann's fluid; P = paraffin; +PC = with postchroming; WB+PE = weak Bouin followed by pyridine extraction; Z = Zenker's fluid; Z3H = Zenker fixation for only 3 hours.

+++ = strong reaction; ++ = moderate reaction; + = weak reaction; — = no observation; O = negative.

The Cytoplasmic Inclusions of the Neurones of *Helix aspersa* and *Limnaea stagnalis*

By J. T. Y. CHOU

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With one plate (fig. 3)

Summary

1. The only cytoplasmic inclusions visible in the living neurone are lipid globules nd mitochondria.

2. In both *Helix* and *Limnaea* there are three kinds of lipid globules, as follows:
(*a*) large globules, yellowish from their carotinoid content;
(*b*) smaller globules, easily coloured by vital dyes;
(*c*) small globules, not colourable by vital dyes.

3. The crescents and rings ('dictyosomes') seen in 'Golgi' preparations appear to e formed by the deposition of silver or osmium on the distorted contents of the maller globules that are easily coloured by the vital dyes.

4. No crescents or rings are seen in the cytoplasm of the living cell.

5. The mitochondria are long, thin threads.

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Introduction

THE purpose of this investigation was to find the form and distribution of the cytoplasmic inclusions in the living neurones of *Helix aspersa* and *imnaea stagnalis*, and to reveal the nature of the bodies commonly called Golgi apparatus', 'Golgi bodies', or 'dictyosomes', which appear when these ells have been treated in particular ways. Several authors (Brambell, 1923; oyle, 1937; Thomas, 1947; Moussa, 1950; and Roque, 1954) have addressed hemselves to these problems, but have not reached agreement among themelves.

Material and Methods

For this investigation, the cerebral ganglia of *Helix aspersa* and *Limnaea tagnalis* were used. The ganglia consist of two groups of neurones which are urrounded by connective tissue; they may easily be separated from this by

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teasing. The groups of neurones were transferred to 0·7 % sodium chlorid solution containing 0·2 % of 10 % anhydrous calcium chloride, slightl flattened under a coverslip, and examined by direct and phase-contra microscopy.

For supravital staining, neutral red chloride, Nile blue, methylene blu brilliant cresyl blue, dahlia violet, and Janus green B were used. The dyes wer dissolved in distilled water and then diluted with saline to the required concentration. The following method was found to give very satisfactory results

Prepare a 1 % solution of the dye in distilled water for use as a stock solution. Before staining add 2 drops of the dye to 2 ml. of the saline solution; th concentration of the dye is now about 0·04 %. Stain a group of neurones i this solution from 10 to 20 minutes, flatten slightly under a coverslip, an examine by direct microscopy.

For intravital staining, 1 ml. of 1 % solution of the dye in saline was injected into the haemocoele. The ganglia were removed after 20 or 30 minute or sometimes after an hour, and mounted in saline for examination. Th animals were always killed by decapitation.

The following methods were used for studying fixed materials:

Sudan techniques

1. Baker's standard Sudan black technique (fixation in formaldehyde saline with special postchroming; gelatine sections, coloration with Suda black B) (Baker, 1944).
2. Sudan black B after fixation in Baker's 'Lewitsky-saline' solution; tha is to say, in Lewitsky's fluid (Flemming-without-acetic) with addition o 0·75 % of sodium chloride (Baker, 1956).
3. Sudan black after fixation in formaldehyde-calcium.
4. Sudan black after simple fixation in formaldehyde-saline.

'Golgi' techniques

1. Weigl's technique (Mann-Kopsch) (Weigl, 1910).
2. Kolatchev's technique (fixation in Champy's fluid, washing in runnin water, post-osmication in 2 % osmium tetroxide at 35° C for 3 days; section were cut at 4 μ and mounted unstained in Canada balsam) (Kolatchev, 1916)
3. Aoyama's silver method (Aoyama, 1929).
4. Fixation in 4 % formaldehyde for 24 hours followed by post-osmication in 2 % osmium tetroxide for 3 days at 35° C (Baker, 1944).

To show mitochondria, ganglia were fixed in Altmann's, Helly's, an Mann's fluids, and sections were stained by Metzner's (1928) and Hirschler' (1927) methods.

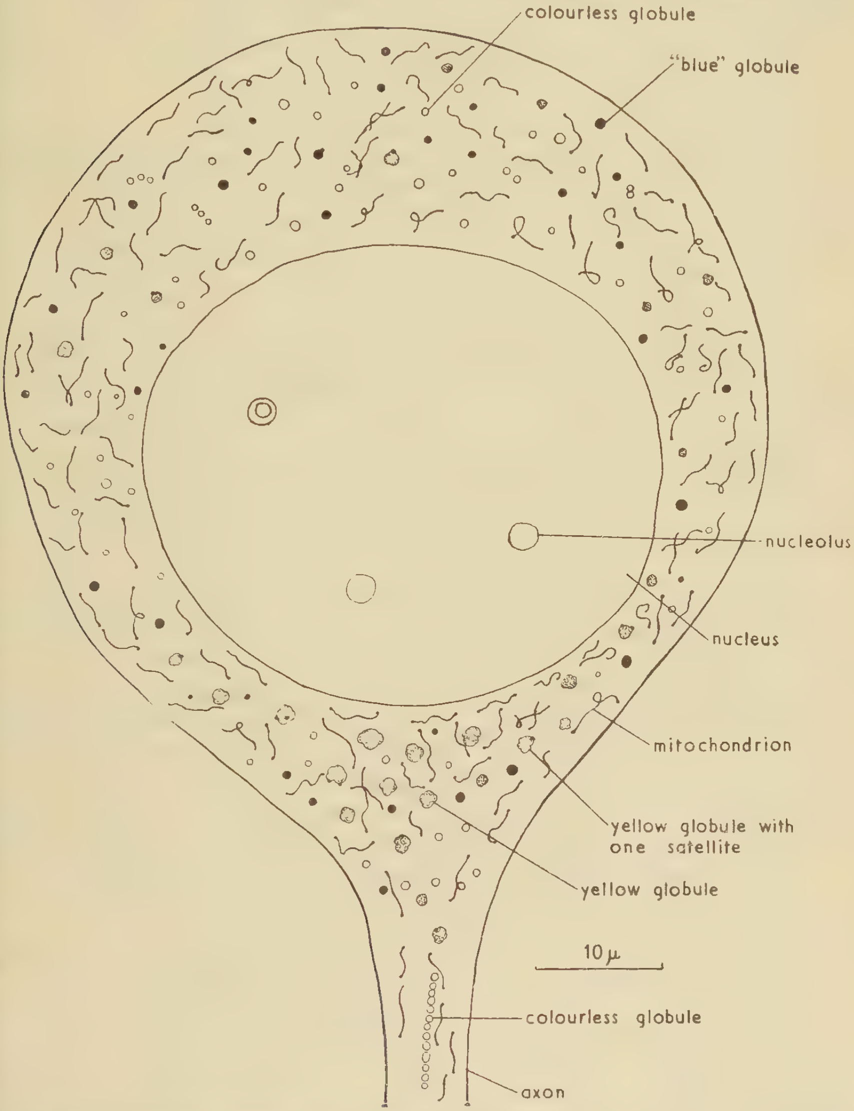
RESULTS

Lipid globules

The unstained living cell

The cells of *Helix aspersa* are unipolar, and about 15 to 70 μ in diameter Each contains a large nucleus with two or three nucleoli. The nucleus fill about two-thirds of the cell (fig. 1).

With direct microscopy, numerous spherical granules varying in diameter rom about 1 to 2 μ can be seen in the cytoplasm. Generally they can be livided into two kinds: one kind is yellowish, large, slightly irregular in shape,



IG. 1. Diagram showing the cytoplasmic inclusions present in the neurone of the cerebral ganglion of *Helix aspersa*.

nd mostly aggregated near the axon hillock. Globules of the second kind are olourless, and in the axon a number of them tend to be arranged like a string f beads. Their diameter is about 1 μ or a little more, but it is never more than ·5 μ. Both of these kinds of globules are rather highly refringent. When examined by phase-contrast microscopy, the globules appear darker than th ground cytoplasm, and homogeneous. One or two small granules (satellite are usually attached to the larger globules of both kinds. Sometimes a larg globule bears three satellites.

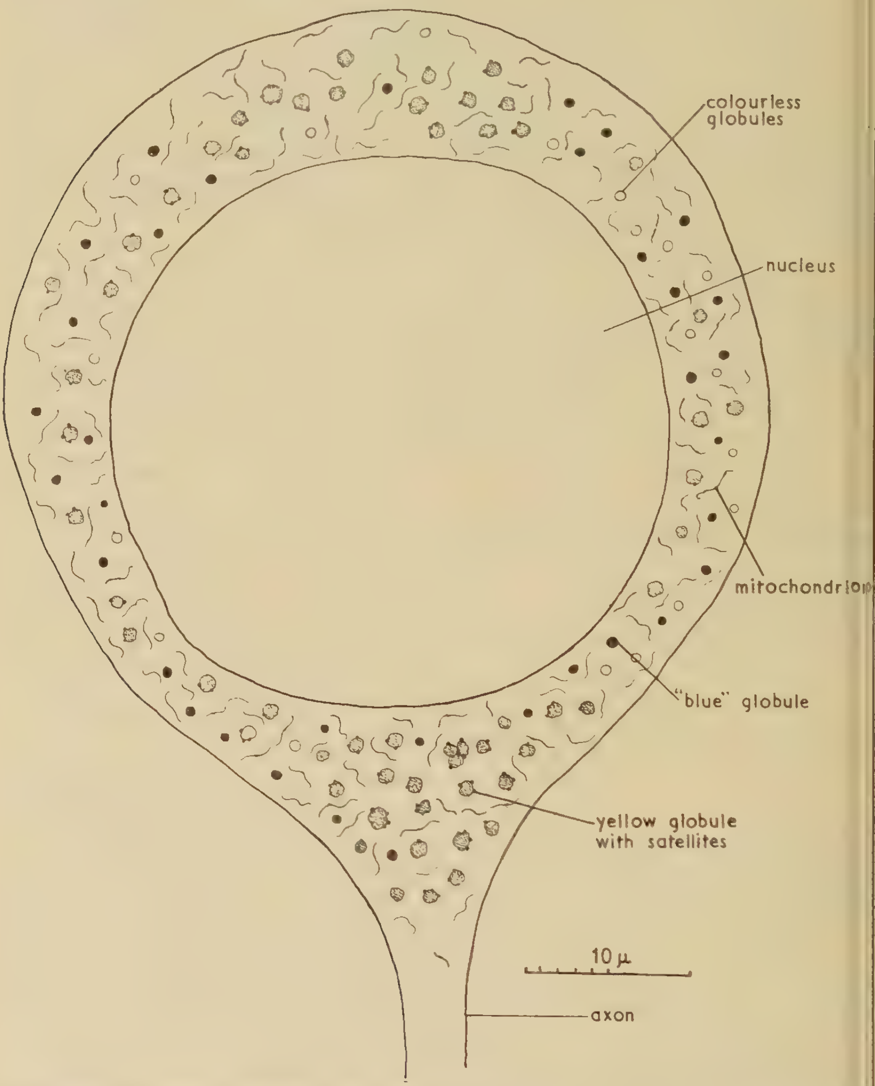


FIG. 2. Diagram showing the cytoplasmic inclusions present in the neurone of the cerebra ganglion of *Limnaea stagnalis*.

In the small neurones the globules are fewer and scattered throughout th cytoplasm with no definite arrangement. The yellowish globules are no present.

The neurones of *Limnaea stagnalis* (fig. 2) have a similar appearance, bu e yellowish globules are of a much deeper yellow and are numerous in both all or large neurones. They are irregular in shape. With phase-contrast icroscopy the irregularity of their form is very obvious. The colourless ones, ually spherical, are not numerous.

There is no sign of any crescent- or cap-shaped bodies in the living cell of ther *Helix* or *Limnaea*.

The vitally stained cell

Nile blue, methylene blue, and brilliant cresyl blue. With *Helix* neurones, these ree dyes all gave exactly similar results. They stained most of the globules, hile the ground cytoplasm, the nucleus, and the remainder of the globules ere unstained. By the use of these dyes the globules in the cytoplasm of *Helix* n be divided into three main groups (fig. 1 and fig. 5, p. 54):

'*Green*' *globules.* These are the same globules that appeared yellowish when amined unstained by direct microscopy. They seem to be formed by aggre- tion of several small ones. They become green after staining for 10 minutes. he satellites, if present, become blue. They usually gather near the axon llock, though small ones are also found in other parts of the cytoplasm. homas (1947) called them 'mulberry spheroids'. Cain (1948) found that rotinoids accumulated in them. The green colour results from the mixture the yellow carotinoid pigment with the blue dye.

'*Blue*' *globules.* These appear homogeneously blue. Their diameter is about to 2 μ. Sometimes they have one or two blue satellites. In small neurones 5 μ) the blue globules are not very numerous. In large neurones (40 to 70 μ) ey occur sometimes in the axon hillock among the green globules.

'*Colourless*' *globules.* A number of globules do not take up any of these three es. Even if the neurones are left in the dye for more than an hour they still main colourless. They are very easily distinguishable in the axon. These lourless globules are seldom more than 1·5 μ in diameter. They are highly fringent. Particular attention was paid to them and it was found that the es in the axon and those in the cytoplasm were indistinguishable.

With Nile blue, but not with other blue dyes, purple globules are occasion- y seen in a few of the cells. There is no means of knowing whether these are bules of a special kind, or whether they are 'blue' globules that have in- rporated a considerable amount of neutral lipid.

Nile blue, methylene blue, and brilliant cresyl blue were also used for intra- al staining. The results were exactly the same as with supravital staining.

Neutral red (*chloride*). Most of the globules were stained reddish by this e, but some of them, especially in the axon, were not coloured. Even after hour they showed no tendency to be stained. The attached granules (satel- es) were always the first to be stained. A similar result was obtained by intra- al staining when 1 % neutral red was injected into the haemocoele.

Dahlia violet. This dye was found not to be useful in this investigation. It oured the ground cytoplasm rather deeply.

Nile blue was used to stain the living neurones of *Limnaea* (fig. 2). The same kinds of globules were seen as in *Helix*. The 'green' globules are very distinc most of them bear blue satellites. Blue globules are fewer than *Helix*; colou less ones fewer still. Pink globules are seen more often than in *Helix*.

Fixed materials

Sudan techniques

A group of neurones of *Helix aspersa* from a very freshly teased cerebr ganglion was transferred, without fixation, to a clean slide with a small amou of saline and attached by slight pressure with a coverslip. They were then p in a saturated solution of Sudan black B in 70 % alcohol for 10 minute differentiated in 70 % alcohol for 5 seconds and in 50 % alcohol for on minute, then washed thoroughly in distilled water, and finally mounted Farrants' medium. All the globules in the neurones became homogeneous blue-black. Thus all the kinds of globules contain lipid, which is evenly distr buted throughout them. There is no differentiation into externum and i ternum.

Baker's standard Sudan black method was used. All the lipid globules a coloured blue-black by this method, and seem to be homogeneous. The yello globules can easily be distinguished from the others, both in form and distribution. It is not possible to distinguish between the globules th stained blue with Nile blue, methylene blue, or brilliant cresyl blue on on hand and those that do not on the other, for all are equally coloured by Suda black (fig. 3, A). The cytoplasm shows weak sudanophilia. Occasionally one two black cap-shaped bodies are seen; perhaps these are formed by the separa tion of the lipid constituents from other substances in the globules as a resu of the action of the fixative.

When tissue is fixed in Lewitsky-saline and embedded in gelatine, and th sections coloured with Sudan black, the globules are seen in very life-li form (figs. 3, B; 4). The yellow globules still retain their form, but are weak coloured by Sudan black; the other kinds of globules are blackened complete

Many crescent- or cap-shaped bodies appear when Sudan colouring agen

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FIG. 3 (plate). Photomicrographs of neurones of *Helix aspersa*. The scale at the bottom appli to all the photomicrographs.

A, Baker's standard Sudan black method, to show the spherical lipid globules.

B, Sudan black B, after fixation in Lewitsky-saline, to show that all the lipid globules are spherical; the yellow globules (y) are weakly positive to Sudan black.

C, Weigl's technique, to show one yellow globule bearing two small satellites (ys), 'Gol dictyosome' (gd) and 'Golgi product' (gp).

D, Sudan IV coloration after Weigl's technique, to show the colourless globule (c) an banana-shaped 'Golgi dictyosome'.

E, Kolatchev technique, showing the 'Golgi dictyosomes'.

F, Aoyama's technique, to show that lipid globules are blackened as thick ring- or ca shaped bodies.

G, fixation in 4 % formaldehyde followed by post-osmication, to show that the result similar to that obtained with Kolatchev's and Aoyama's method.

H, a very large neurone fixed in Mann's fluid and stained with Metzner's acid fuchsin, show the fine, thread-like mitochondria with granular ends (m).

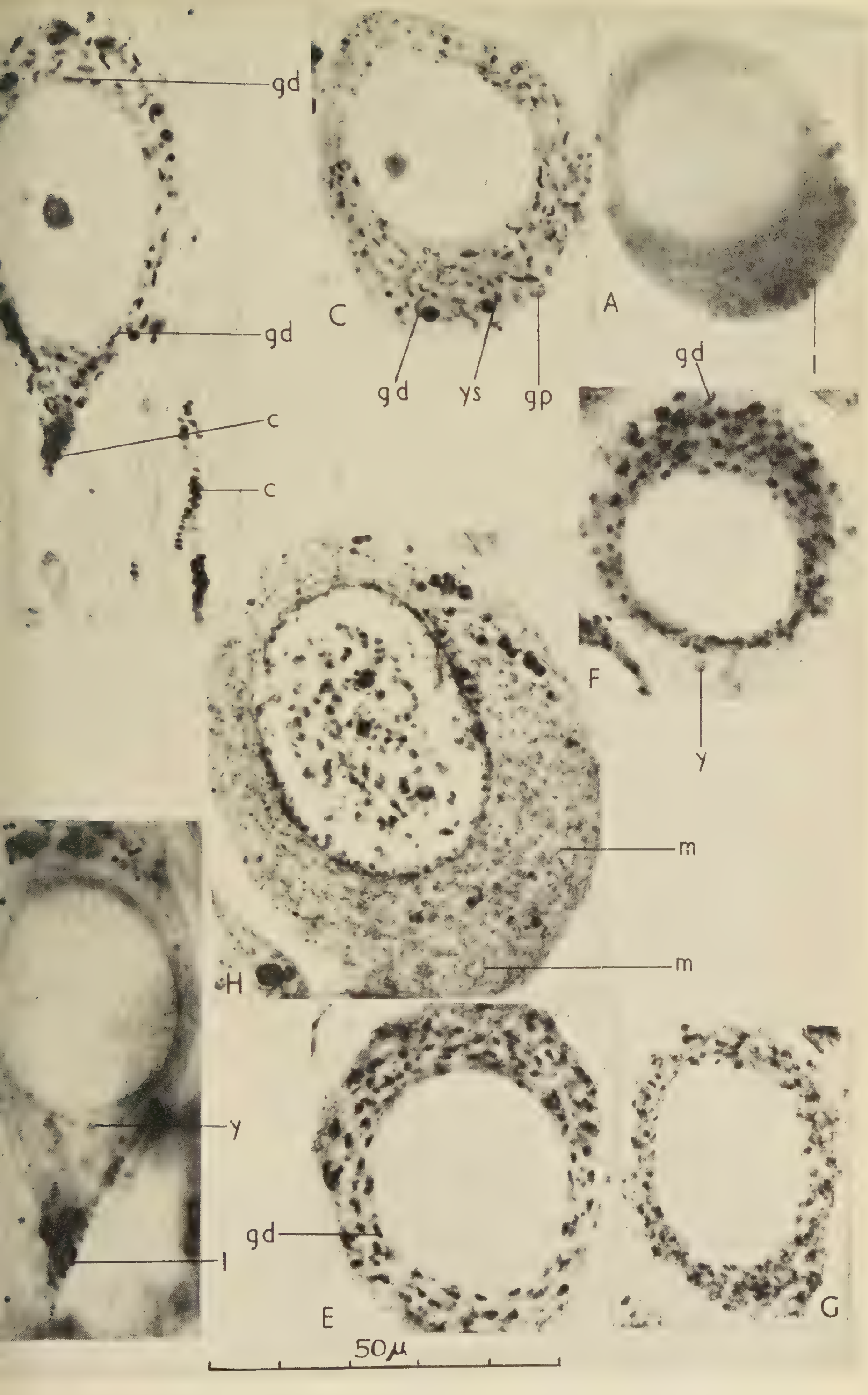


FIG. 3

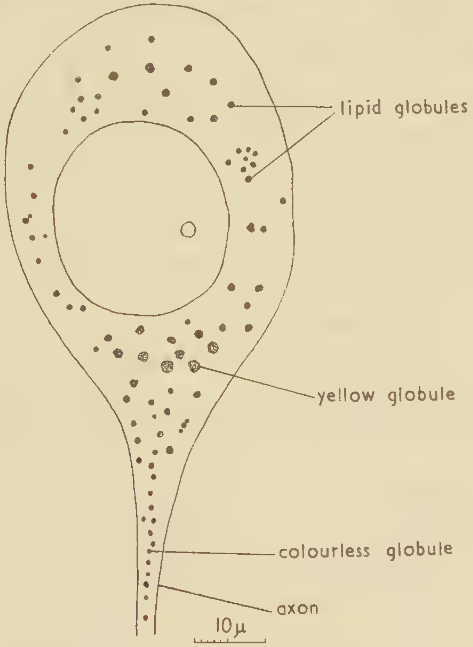
J. T. Y. CHOU

re used after fixation in formaldehyde-saline or formaldehyde-calcium withut post-chroming.

In general, the results obtained with *Limnaea* by the use of Sudan black reemble those obtained with *Helix*. The yellow globules, however, colour more trongly, and the other globules have a tendency to become slightly irregular n shape. Spherical vacuoles, untouched by Sudan black, are sometimes seen; hese appear to be artificial, and are not often seen in tissue fixed with Lewitsky-saline.

'Golgi' techniques

It will be convenient to describe first the results of the application of the Weigl (Mann-Kopsch) technique to the neurones of *Helix* (fig. 3, c). This can e divided into two stages: (1) fixation by Mann's fluid, and (2) post-smication.



IG. 4. Diagram of a neuron of *Helix aspersa* to show the position of lipid globules after fixation in Lewitsky-saline and coloration with Sudan black.

Fig. 5 shows the three kinds of globules stained with Nile blue or methyne blue in the living cell.

The effect of fixation alone is shown in fig. 6. Ganglia were fixed in Mann's uid and embedded in paraffin without post-osmication. Sections were oloured with Sudan black or Sudan IV and mounted in Farrants' medium.

Yellow globules (fig. 6, A). The shape is scarcely changed from the living contion. The globule is pale grey, the satellites somewhat reduced in size and arker.

'*Blue globules*' (fig. 6, B). These are much changed in appearance. In optica section one sees a crescent (blue-black with Sudan black, red with Sudan IV pressed against one side of the globule, the rest being very pale grey like th cytoplasm. The satellites are not so clearly seen as in life.

Colourless globules (fig. 6, C). These are more changed in external form tha the other globules, as the surface is no longer smooth. They are blue-black o red, very pale in the centre.

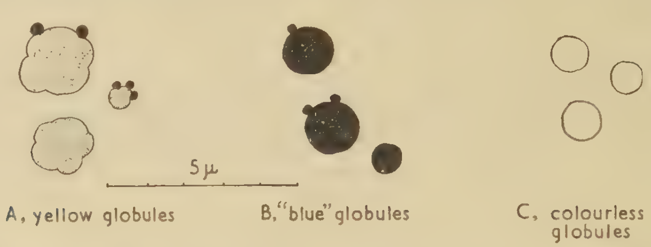


FIG. 5. Diagrams showing the lipid globules stained supravitally with Nile blue, methylen blue, or brilliant cresyl blue.

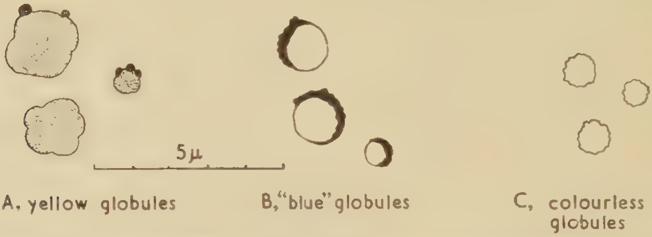


FIG. 6. Diagrams showing the change of form of the lipid globules caused by a 'Golgi' fixativ (Mann's), without silvering or post-osmication.

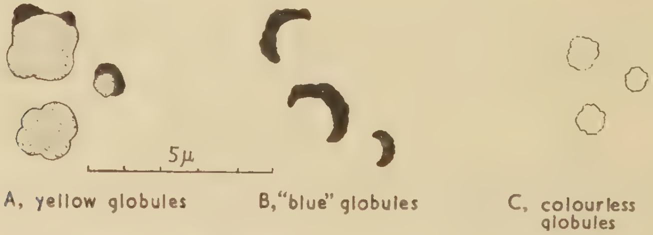


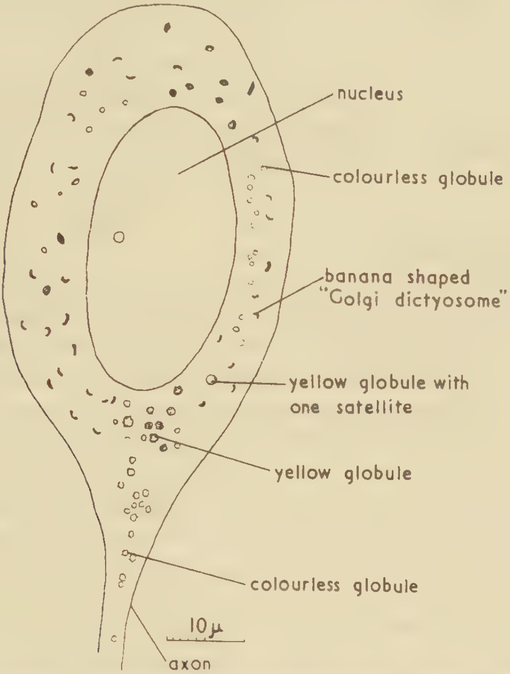
FIG. 7. Diagrams of lipid globules as they appear in Weigl preparations.

The effect of the application of the whole of the Weigl technique is show in fig. 7. A comparison of this with fig. 6 shows the result of post-osmication.

Yellow globules (fig. 7, A). The appearance is scarcely changed except tha there is a heavy black deposit of osmium round the satellites. When there ar two or more satellites, a deposit of osmium may cover and connect them. Th whole globule now looks duplex, as though it had an internal and incomplet external part.

'*Blue*' *globules* (fig. 7, B). The object appearing crescentic in optical sectio is now black, but the osmium has been deposited in such a way as to thicken the outside of it. Satellites are sometimes enclosed by the deposit of osmium. The external surface of the deposited osmium is somewhat irregular. The rest of the globule is not distinguishable from the cytoplasm.

Colourless globules (fig. 7, C). These are scarcely seen in Weigl preparations, but subsequent colouring with Sudan IV shows them clearly by making them evenly red all through (figs. 3, D; 8).


nucleus
colourless globule
banana shaped "Golgi dictyosome"
yellow globule with one satellite
yellow globule
colourless globule
10μ
axon

IG. 8. Diagram of a neurone of *Helix aspersa* to show how Weigl's technique changes the orm of lipid globules. The colourless globules make their appearance only after coloration with Sudan IV.

With the Kolatchev technique it is difficult or impossible to distinguish he three kinds of globules. There is a tendency for the osmium to be deposited ver the greater part of the globules so that rings as well as crescents are seen optical section (fig. 3, E). If the sections are bleached and coloured with udan black, the yellow globules are easily distinguished by their form; they ppear grey.

The technique of Aoyama causes silver to be deposited over nearly the hole surface of the globules, so that many of them appear as rings in optical ection. Irregular crescents, not so sharply formed as in Weigl preparations, re also seen. The silver makes a much thicker and more irregular deposit han the osmium. The yellow globules can be distinguished by their larger ze and clear interior (fig. 3, F). When a ganglion is fixed and silvered by oyama's method and frozen sections are coloured with Sudan IV, some of the globules (especially the 'colourless' ones in the axon, and the yellow globules) show a reddish internal part and a black silver deposit on the surface.

When tissue was fixed in 4 % formaldehyde and post-osmicated, the result was similar to that obtained with Kolatchev's and Aoyama's techniques (fig. 3, G).

Weigl's technique was applied to ganglia of *Limnaea*. The yellow globules can easily be distinguished from the others, because they retain their irregularly globular shape, while the other globules are transformed into 'crescents'. The osmium is deposited on the satellites of the yellow globules and on the surface of the yellow globules in the vicinity of the satellites, so that a crescentic appearance is given in an optical section. A greater part of the surface of the yellow globules is covered with osmium than in a *Helix* preparation. The 'colourless' globules cannot be distinguished.

Mitochondria

The unstained living cell

With phase-contrast microscopy, the mitochondria in the neurone of *Helix aspersa* are seen as numerous, fine filaments in the cytoplasm; in the axon they tend to be arranged parallel to its axis (fig. 1). Their length is between 5 and 8 μ. They are blunt-ended, and often have a granule at one or both ends. These filaments are similar in small and large neurones. They are more easily seen in hypertonic than in isotonic solution, but in the former they disappear in an hour or so.

The mitochondria of *Limnaea stagnalis* are exceedingly fine, curled filaments. Sometimes the thread is slightly thickened somewhere along its length. There is never a granule at the end, as in *Helix*.

The vitally stained cell

Janus green B. Satisfactory results were not obtained with this dye in the living cell of either *Helix* and *Limnaea*.

Fixed materials

Ganglia were fixed in Helly's, Altmann's, and Mann's fluid and stained by Metzner's (1928) acid fuchsin and Hirschler's (1927) iron haematoxylin.

The mitochondria of *Helix* appear as very fine filaments, exactly as seen in the living cell; their granular ends are perhaps even more distinct (fig. 3, H). A number of spherical or cap-shaped fuchsinophil bodies showed after Metzner's technique. These structures seem to be identical with the 'blue' globules in their distribution.

In *Limnaea stagnalis* the mitochondria appeared as fine, curled filaments. Granular mitochondria were only very rarely seen.

DISCUSSION

The appearance called 'Golgi apparatus', 'Golgi bodies', or 'dictyosomes' in these cells results from the artificial modification of lipid globules that are easily visible in the living cell. Osmium has a strong tendency to be deposited on the surface of the yellow globules, especially in the vicinity of the satellites. The 'blue' globules give a different appearance. The lipid constituent tends to be thrown by the fixative against the edge of the globule on one side, and osmium then accumulates in this artificially produced 'crescent' and on its surface. The 'colourless' globules do not appear to give rise to any Golgi artifact.

It is not impossible that the cytoplasm in the immediate vicinity of the yellow and 'blue' globules may be in some way different from the rest of the cytoplasm at the submicroscopic level, but none of the techniques used in this study has suggested the presence of any kind of special structure round the globules.

It remains to compare the conclusions reached by myself with those reached by other students of the same cell.

My phase-contrast view of the cell agrees well with that of Roque (1954). I differ from him only in classifying the globules by their reactions to vital dyes and not by their sizes. Roque did not investigate the origin of the Golgi artifact. Brambell (1923) made silver preparations resembling my own in general, but did not attempt to identify in the living cell the objects responsible for the reaction of the silver. Boyle (1937) studied the living cell and saw the globules colour with neutral red. He noticed that some of the globules did not colour with neutral red or methylene blue. He considered these to be metamorphosed mitochondria. I have found no support for this view of the origin of the 'colourless' globules. He also showed that in frozen sections of material treated by Aoyama's method, globules became coated by silver on the surface while the interior could be coloured by Sudan IV. He neither stated nor denied there was any relation between the globules seen in the living cell and the crescents and rings seen in the osmium and silver preparations. Thomas's (1947) 'mulberry spheroids' clearly correspond with my yellow globules. He considered that the crescents seen in osmium preparations are in fact over-impregnated mitochondria. He partially bleached Mann-Kopsch preparations and found that the 'dictyosomes', now greatly reduced in thickness, could usually be resolved into a row of granules, which he referred to as a mitochondrion. I do not agree with him, because the mitochondria are so numerous that if they were over-impregnated to produce 'dictyosomes' one could hardly see the spaces between them. The objects he saw were probably 'blue' globules, distorted by the Golgi technique.

Moussa (1950) studied the neurones of *Limnaea stagnalis*. He noticed that certain globules in the cytoplasm, which he called 'lipochrome globules', are naturally coloured golden yellow, and that of the other globules some take up vital dyes while others do not. This is in agreement with my findings. He claimed to have seen reddish-brown crescents or rims in the golden yellow globules in living cells. This is not in conformity with my findings. Moussa considered that the crescents or rims become separate from the internal part of the globules. The latter constitute the 'Golgi product'. The crescents and rims now become invisible in life, even by phase-contrast microscopy, but can be silvered or osmicated to form the 'Golgi dictyosomes'. I have seen nothing that would suggest that this is a true explanation of the origin of the curved black objects seen in a Golgi preparation.

An important difference between Moussa and myself is that he derives the curved black objects (Golgi bodies or dictyosomes) from the yellow globules, whereas I derive them from the 'blue' globules. I do not find it necessary to postulate the existence of any totally invisible objects large enough to be seen easily by phase-contrast microscopy.

Young (1932, 1953, 1956) has remarked that the neutral red globules in the neurones of cephalopods can be swollen or shrunk osmotically, and he concludes that they might be bounded by a semi-permeable membrane. Such a membrane, however, could not be seen by electron microscopy. I have not seen any indication of a membrane in my studies of the lipid globules of *Helix* and *Limnaea*.

My observations on *Helix* entirely agree with those of Roque (1954). Thomas (1947) found that in fixed preparations the threads were often broken up into strings of granules ('coccoids'). Brambell and Gatenby (1923) and Boyle (1937) described the mitochondria as granules. This is certainly not so in the living cell.

Moussa (1950) described the mitochondria of *Limnaea* as granular, or sometimes as consisting of rows of granules. This is not true of the living cell.

I wish to express my gratitude to Dr. J. R. Baker for suggesting and supervising this investigation; to Professor A. C. Hardy, F.R.S., for providing me with facilities for working in his Department; to Mr. S. Bradbury and Miss B. M. Jordan for advice on certain matters; and to Mr. P. A. Trotman for supplying me with animals.

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REFERENCES

Aoyama, F., 1929. Z. wiss. Mikr., **46**, 489.
Baker, J. R., 1944. Quart. J. micr. sci., **85**, 1.
—— 1956. Personal communication.
Boyle, W., 1937. J. Roy. micr. Soc., **57**, 243.
Brambell, F. W. R., 1923. J. Physiol., **57**, 415.
Brambell, F. W. R., and Gatenby, J. B., 1923, quoted by Boyle, W., 1937, J. Roy. micr. Soc., **75**, 243.
Cain, A. J., 1948. Quart. J. micr. sci., **88**, 383.
Hirschler, J., 1927. Z. wiss. Mikr. **44**, 216.
Kolatchev, A., 1916. Arch. Russes. d'Anat. d'Hist. d'Emb., **1**, 383.
Metzner, R., and Krause, R., 1928. In Abderhalden's *Handbuch der biologischen Arbeitsmethoden*, Abt. V, Teil 2, I Hälfte, 325.
Moussa, T. A. A., 1950. J. Morph., **87**, 27.
Roque, A. L., 1954. J. Roy. micr. Soc., **74**, 188.
Thomas, O. L., 1947. Quart. J. micr. Sci., **88**, 445.
Weigl, R., 1910. Bull. Internat. Acad. Soc. Cracovie, Sér. B (no vol. number), 691.
Young, J. Z., 1932. Quart. J. micr. sci., **75**, 1.
—— 1953, Ibid., **94**, 399.
—— 1956. Endeavour, **15**, 5.

The Chemical Composition of Lipid Globules in the Neurones of *Helix aspersa*

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SUMMARY

1. The three kinds of globules that can be recognized in the living neurone of *Helix aspersa* are sharply distinguishable from one another by histochemical tests.

2. One kind of globule contains phospholipid; another appears to consist of triglyceride; the third is complex chemically, containing mixed lipids with some protein and carbohydrate.

3. When osmium techniques for the 'Golgi apparatus' are applied to this particular cell, phospholipid is blackened.

IN an earlier paper (Chou, 1957) it was shown that there are three kinds of lipid globules in the cytoplasm of the neurones of *Helix aspersa*. They are shown here in fig. 1. These three kinds react quite differently to vital dyes. One kind is readily coloured blue by Nile blue, methylene blue, and brilliant cresyl blue: I therefore call these 'blue' globules, though they are colourless in life. Another kind cannot be coloured by any vital dye, so far as is known: I call these colourless globules. The third kind is in life pale yellow, and I call these yellow globules. They appear green when coloured by the blue vital dyes. The colourless and yellow globules are more refringent in life than the 'blue' ones. The purpose of the present investigation was to find whether there were histochemical differences between the three kinds of globules.

In fixed preparations it is easy to distinguish the colourless globules because many of them lie in nearly straight rows at the base of the axon. The yellow globules are generally the largest; they are irregular in shape, and many of them are grouped in or near the axon hillock, though they are not confined to this region. The 'blue' globules are recognized by their being spherical, nearly uniformly distributed throughout the cytoplasm, and different in their reactions from the colourless globules.

The techniques used are set out in tabular form in the appendix (p. 64). The cerebral ganglion was used, or sometimes the sub-oesophageal ganglion-mass. The neurones of all the ganglia are similar in their cytoplasmic inclusions, though there is considerable difference in size.

RESULTS

All three kinds of globules react positively to Sudan IV and Sudan black. It is clear that they contain or consist of lipid.

None of the three kinds reacts positively to Fischler's test for fatty acids,

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Windaus's for cholesterol, or Sakaguchi's for arginine, &c. The globules are not basiphil nor chromotropic. Feulgen's test is negative.

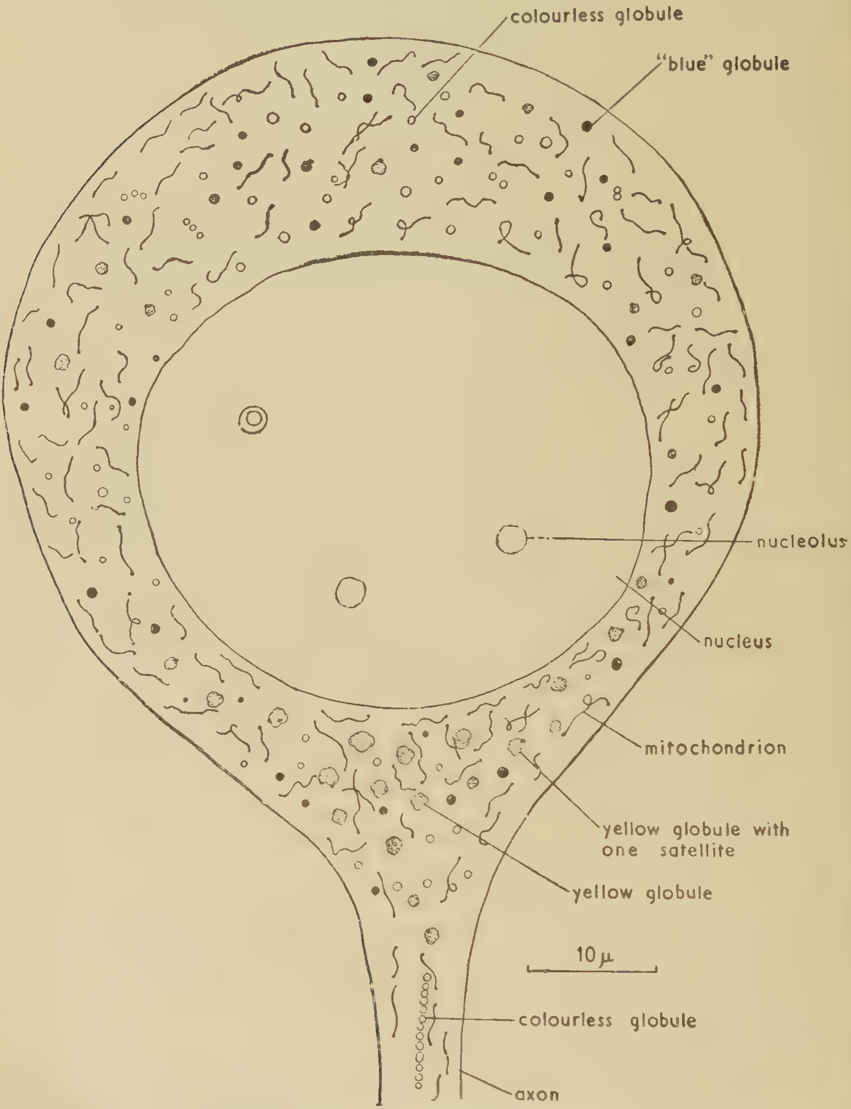

FIG. 1. Diagram showing the cytoplasmic inclusions present in the neurones of the cerebral ganglion of *Helix aspersa*. From Chou (1957).

The three kinds of globules are distinguishable by the tests enumerated below.

'*Blue*' *globules*. These are the only globules that react positively to the acid haematein (AH) test. The test was negative after pyridine extraction. The globules therefore contain phospholipid. When Metzner's method for mitochondria is applied, the acid fuchsine dyes the globules strongly. There is often a correlation between red staining with Metzner's method and the presence of phospholipid.

Apart from the Sudans and AH, no histochemical test used in this investigation gave a positive result with the 'blue' globules.

The low refringence of these globules suggests that the phospholipid may be associated with water.

Colourless globules. These are negative to all the tests except the Sudans (Ciaccio's test is positive). The facts suggest but do not prove that the globules contain triglyceride. The negative result with Nile blue (neither blue nor red reaction) is difficult to interpret. It is unfortunate that there is no positive test by which the presence of the triglycerides can be proved. The high refringence of these globules suggests that they may consist entirely of lipid.

Yellow globules. These are the only globules that have a non-lipid as well as a lipid content. They are considerably more complex histochemically than the 'blue' and colourless globules.

Cain (1948) showed, by the application of concentrated sulphuric acid, that the pigment in these globules is carotenoid. This was confirmed also in the present investigation by the Carr–Price reaction (Carr and Price, 1926), but the reaction was feeble. The globules are of a much paler colour than the corresponding ones in *Limnaea stagnalis* and *Planorbis corneus* (Cain, 1948). AH gives a feeble reaction on the surface only of the globules. The test for plasmalogen is positive. Liebermann's test for cholesterol and its esters is positive (though weakly), provided that the sections are exposed to as much direct sunlight as possible for about 10 days. (Oxidation by iron alum is not successful.) The positive reactions with the PFAS and PAAS tests suggest unsaturation of the lipids.

The periodic acid / Schiff test gives a positive result. After digestion with saliva the reaction is weaker (though it still exists). This suggests the presence of a carbohydrate. If a ganglion is fixed in cold acetone and sections are coloured with Sudan black, the globules become pale grey; this is not so if hot acetone in a Soxhlet apparatus is used. The facts suggest the presence of cerebroside (Casselman and Baker, 1955), and would account in part for the positive PAS reaction. There is no evidence of the existence of mucopolysaccharides, acidic or netural.

The Hg/nitrite test (Baker, 1956) gives a weakly positive result, presumably indicating the presence of tyrosine in protein; but, as remarked before, the Sakaguchi test gave a negative result. There is not enough protein to leave a stainable 'granule' in fixed preparations.

No general enzymological study was undertaken, but Gomori's (1952) test for alkaline phosphatase was performed. This gave a positive result on the surface of the yellow globules; the latter were covered with tiny black dots. It is interesting to notice that the phosphatase appears in the same position as phospholipid.

DISCUSSION

It is clear that the three kinds of globules distinguishable in life in the neurones of *Helix aspersa* are sharply different in chemical composition. The facts may be briefly summarized thus:

'Blue' globules. These contain phospholipid.

Colourless globules. These probably consist of triglyceride.

Yellow globules. These are complex chemically. They appear to contain mixed lipids, with some carbohydrate and protein; the colour is due to carotenoid.

In osmium preparations for the 'Golgi apparatus', the phospholipid of the 'blue' globules is precipitated by the fixative on one side of the sphere, and then blackened by reduced osmium. The resulting crescent- or cap-shaped body is commonly called a 'dictyosome' (net-body). Osmium also blackens the surface of the yellow globules, and indeed Moussa (1950), in his description of the neurones of *Limnaea stagnalis*, regards the blackened surface of the yellow globules as the 'dictyosome' and the rest of the globule as 'Golgi product'.

The phospholipid of the yellow globules is situated at their surface in fixed preparations; this was shown by Thomas (1947) and confirmed in the present investigation. It is not shown whether during life the phospholipid is at the surface or distributed throughout the globule.

It is of interest to note that in these cells osmium tends to be deposited, in 'Golgi' preparations, wherever there is an accumulation of phospholipid. In the neurones of mammals osmium is deposited in the form of the characteristic Golgi 'network', but no one has described a phospholipid network that could be responsible for this appearance. It seems best to use ordinary chemical nomenclature whenever possible, in preference to ill-defined words like 'Golgi apparatus'. Lipid globules do indeed occur in the neurones of mammals, and these contain phospholipid (Casselman and Baker, 1955). They may perhaps be the same as the *plaquettes* seen by Golgi (1898) in his network.

In Aoyama preparations silver is deposited on the surfaces of all three kinds of lipid globules.

I have great pleasure in acknowledging my debt to Dr. J. R. Baker for suggesting and supervising this investigation, and to Professor A. C. Hardy, F.R.S., for providing me with facilities for working in his department.

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REFERENCES

BAKER, J. R., 1944. Quart. J. micr. Sci., **85**, 1.
—— 1946. Quart. J. micr. Sci., **87**, 44.
—— 1947. Ibid., **88**, 115.
—— 1949. Ibid., **90**, 293.
—— 1956. Ibid., **97**, 161.

IGNARDI, C., 1940. Boll. Soc. ital. Biol. sperim., **15**, 594.
AIN, A. J., 1947. Quart. J. micr. Sci., **88**, 383.
—— 1948. Ibid., **89**, 421.
ARR, F. H., and PRICE, A., 1926. Biochem. J., **20**, 497.
ASSELMAN, B. W. G., and BAKER, J. R., 1955. Ibid., **96**, 46.
HOU, J. T. Y., 1957. Ibid., **98**, 47.
EULGEN, R., and ROSSENBECK, H., 1924. Z. phys. Chem., **135**, 203.
OLGI, C., 1898. Arch. ital. de Biol., **30**, 60.
OMORI, G., 1952. *Microscopic histochemistry.* Chicago (University Press).
ERXHEIMER, G. W., 1901. Deut. med. Woch., **36**, 607.
ILLIE, R. D., 1952. Stain Tech., **19**, 55.
ISON, L., 1953. *Histochimie et cytochimie animales.* Paris (Gauthier-Villars).
ETZNER, R., and KRAUSE, R., 1928. In Abderhalden's *Handbuch der biologischen Arbeitsmethoden*, Abt. V, Teil 2, I Hälfte, 325.
OUSSA, T. A. A., 1950. J. Morph., **87**, 27.
EARSE, A. G. E., 1951. Quart. J. micr. Sci., **92**, 4.
—— 1954. *Histochemistry, theoretical and applied.* London (Churchill).
HOMAS, O. L., 1947. Quart. J. micr. Sci., **88**, 445.

APPENDIX

A summary of the histochemistry of the lipid globules in the neurones of Helix aspersa

Test applied					*Results obtained with the three kinds of lipid globul*		
Name of test	*Fixation*	*Embedding medium*	*Thickness of sections in μ*	*Reference*	*Yellow globules*	*'Blue' globules*	*Colourless globules*
Standard Sudan black	FCa+PC Fs+PC	G	10	Baker, 1944, 1949	+++	+++	+++
Sudan IV	FCa+PC Fs+PC	G	10	Herxheimer, 1901	+++	+++	+++
Windaus's	Fs; FCa	G	10	Lison, 1953	O	O	O
Liebermann's	Fs	G	10	Lison, 1953	+	O	O
Fischler's	F; FCaS	G	10	Pearse, 1954	O	O	O
Acid haematein	FCa+PC	G	10	Baker, 1946	+	++	O
Acid haematein, pyridine extraction	WB+PE	G	10	Baker, 1946	O	O	O
Ciaccio's	CF+PC (6 days)	P	8	Lison, 1953	+++	+++	+++
PAAS	Z	P	8	Pearse, 1954	++	O	O
PFAS	Z	P	8	Pearse, 1954; Lillie, 1952	++	O	O
Casselman and Baker's for cerebroside	CA	G	10	Casselman and Baker, 1955	+	O	O
Casselman and Baker's for cerebroside, control	HA	G	10	Casselman and Baker, 1955	O	O	O
Nile blue	FCa	G	10	Cain, 1947	O	O	O
PAS	Z	P	8	Pearse, 1954	+++	O	O
PAS, control	Z	P	8	Pearse, 1954	O	O	O
PAS after saliva digestion	Z	P	8	Pearse, 1954	++	O	O
Feulgen's	Z	P	8	Feulgen and Rossenbeck, 1924	O	O	O
Feulgen's, control	Z	P	8	Feulgen and Rossenbeck, 1924	+	O	O
Plasmal	freshly teased			Pearse, 1954	+	O	O
Cain's for carotenoids	freshly teased			Cain, 1948	+	O	O
Carr and Price test	freshly teased			Carr and Price, 1926	+	O	O
Metachromasy	Z	P	8	—	O	O	O
Bignardi's for neutral polysaccharide	H	P	8	Bignardi, 1940	O	O	O
Sakaguchi	Z	P	8	Baker, 1947	O	O	O
Hg/nitrite	Fs	C	15	Baker, 1956	+	O	O
Gomori's for alkaline phosphotase	Alc/Ac	freshly teased		Gomori, 1952	+	O	O
Metzner's	Alt	P	4	Metzner and Krause, 1928	+	++	O
Basiphilia	Z	P	8	—	O	O	O

KEY: Alc/Ac = absolute alcohol / acetone mixture; Alt = Altmann's fluid; C = celloidin; CA = cold acetone; CF = Ciaccio's fixative; F = neutral formaldehyde; FCa = formaldehyde calcium; FCaS = neutral formaldehyde with saturated calcium salicylate; Fs = formaldehyde saline; G = gelatine; H = Hermann's fluid; HA = hot acetone; P = paraffin. +PC = with postchroming; WB+PE = weak Bouin followed by pyridine extraction; Z = Zenker's fluid; +++ = strong reaction; ++ = moderate reaction; + = weak reaction; O = negative.

Cytoplasmic Inclusions of the Neurones of Gastropods

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SUMMARY

The neurones of the sub-oesophageal ganglionic mass of the Simla slug, *Anadenus altivagus*, and the Bharwain snail, *Euaustenia cassida*, have been investigated by phase-contrast microscopy of the living cells and in fixed preparations. The mitochondria are seen as granules and filaments in both living and fixed material. Alignment of granules into filamentous mitochondria has also been observed in the fixed preparations. The lipid spheroids (corresponding to the 'binary spheroids' of Thomas and the 'lipochondria' of Roque) are sudanophil, osmiophil, and argentophil. The bigger spheroids show a duplex structure, consisting of a cortical, chromophil, lipid component, which may be in the form of a complete ring (*Anadenus*) or in the form of one or two granules or a crescent (*Anadenus* and *Euaustenia*), and a chromophobe medulla (neutral red vacuome of Parat), in which lipochrome develops to form the 'mulberry spheroids' of Thomas. The small homogeneous lipid spheroids also contribute to the formation of mulberry spheroids'.

THE spheroid bodies (often called 'Golgi bodies') in the cytoplasm of neurones have been studied by many authors, especially in *Helix* and other gastropods. Agreement has not, however, been reached as to the structure and homologies of these bodies. It was thought desirable to investigate the cytoplasmic inclusions of the neurones of gastropods belonging to genera not previously examined in this respect.

MATERIAL AND TECHNIQUE

The slugs, *Anadenus altivagus* Theobold, were collected at Simla, a hill station in the Panjab (India), during the month of August 1955. Living neurones were studied by phase-contrast microscopy at Simla. Some material was also fixed there and brought down to Hoshiarpur, where it was sectioned and studied.

Living specimens of the snail, *Euaustenia cassida* Hutton, were collected at Bharwain, a village in the lower Shiwalik Range in Hoshiarpur District during he month of October 1955, and were brought to Hoshiarpur.

The neurones of the sub-oesophageal ganglionic mass of *Anadenus* and the pleuro-pedal ganglionic mass of *Euaustenia* were selected for the present investigations.

The living neurones of *Anadenus* were studied in saline solution (Baker, 944) under a Zeiss phase-contrast microscope (stand 'W').

The following fixatives were employed:

Formaldehyde—osmication. Material fixed in formaldehyde saline was osmicated in 1% solution of osmium tetroxide in saline at 37° C for 24 to 48 ours. The sections were cut by Peterfi's cellodin methyl-benzoate method, ecommended by Baker (1944). Some of the sections were bleached in a aturated solution of potassium persulphate before study.

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Aoyama. The material fixed in cadmium formaldehyde mixture was treate with silver nitrate for 15 to 24 hours and reduced for 4 to 6 hours. Some of th Aoyama preparations were toned in gold chloride solution.

Altmann. The sections of material fixed in Altmann were stained in iro haematoxylin for the study of mitochondria. Some of the sections were studie unstained immediately after mounting to locate the lipid contents.

Helly with postchroming. The material fixed in Helly's fixative was postchromed at 37° C in a saturated solution of potassium dichromate for 4 hours. The paraffin sections were coloured in a saturated solution of Suda black in 70% alcohol, and mounted in Farrants's medium (Thomas, 1948 Some of the sections were also stained with Sudan IV. Some of the section were stained in Masson's tricolour stain.

The sections of this material were used for the study of mitochondria afte staining in iron haematoxylin.

OBSERVATIONS

The sub-oesophageal ganglionic mass of *Anadenus altivagus* and *Euausteni cassida* is formed by the fusion of a number of ganglia, which cannot be completely separated. It is, therefore, very difficult, if not impossible, to say definitely to which particular ganglion the neurones segregated for study reall belong.

MITOCHONDRIA

The mitochondria could best be studied in Helly and Altmann material stained with 0·5% iron haematoxylin. They could also be observed in formaldehyde-osmicated slides that had been bleached and subsequently staine with iron haematoxylin (Gatenby and Moussa, 1949; Moussa, 1950), but suc preparations were not found to be very satisfactory. In Sudan black preparations the mitochondria are seen as greyish bodies.

The mitochondria are in the form of granules and filaments in the neurone of both species. They are stained dark grey by haematoxylin and pink by aci fuchsin. The filamentous mitochondria are few in number and are seen as fin curled threads with a uniform contour, scattered here and there amongst th uniformly dispersed granular ones (figs. 1, A–C; 3, A). These truly filamentou mitochondria can be easily distinguished from filaments formed by the alignment of granular mitochondria in fixed preparations (figs. 1, A; 3, A, E). Th latter could be compared with the 'coccoids' of *Helix* neurones (Thomas 1947).

FIG. 1. Neurones of *Anadenus*. A, Helly / haematoxylin, showing mitochondria and sma homogeneous spheroids. B, Helly / Masson, showing fuchsinophil granules forming 'mul berry spheroids'. Duplex and homogeneously stained bigger spheroids are also seen. C, Helly haematoxylin, showing mitochondria, homogeneously stained spheroids, and 'mulberr spheroids'. D and E, formaldehyde-osmicated, showing some of the duplex spheroids wit incomplete cortex having partially or completely collapsed chromophobe medulla. Variou forms of spheroids, including 'mulberry spheroids', are also seen. F, Aoyama (toned), smalles observed neurone. G, Aoyama (toned), showing the formation of 'rods'. H, Aoyama, showin 'mulberry spheroids' and thick cortices of the spheroids.

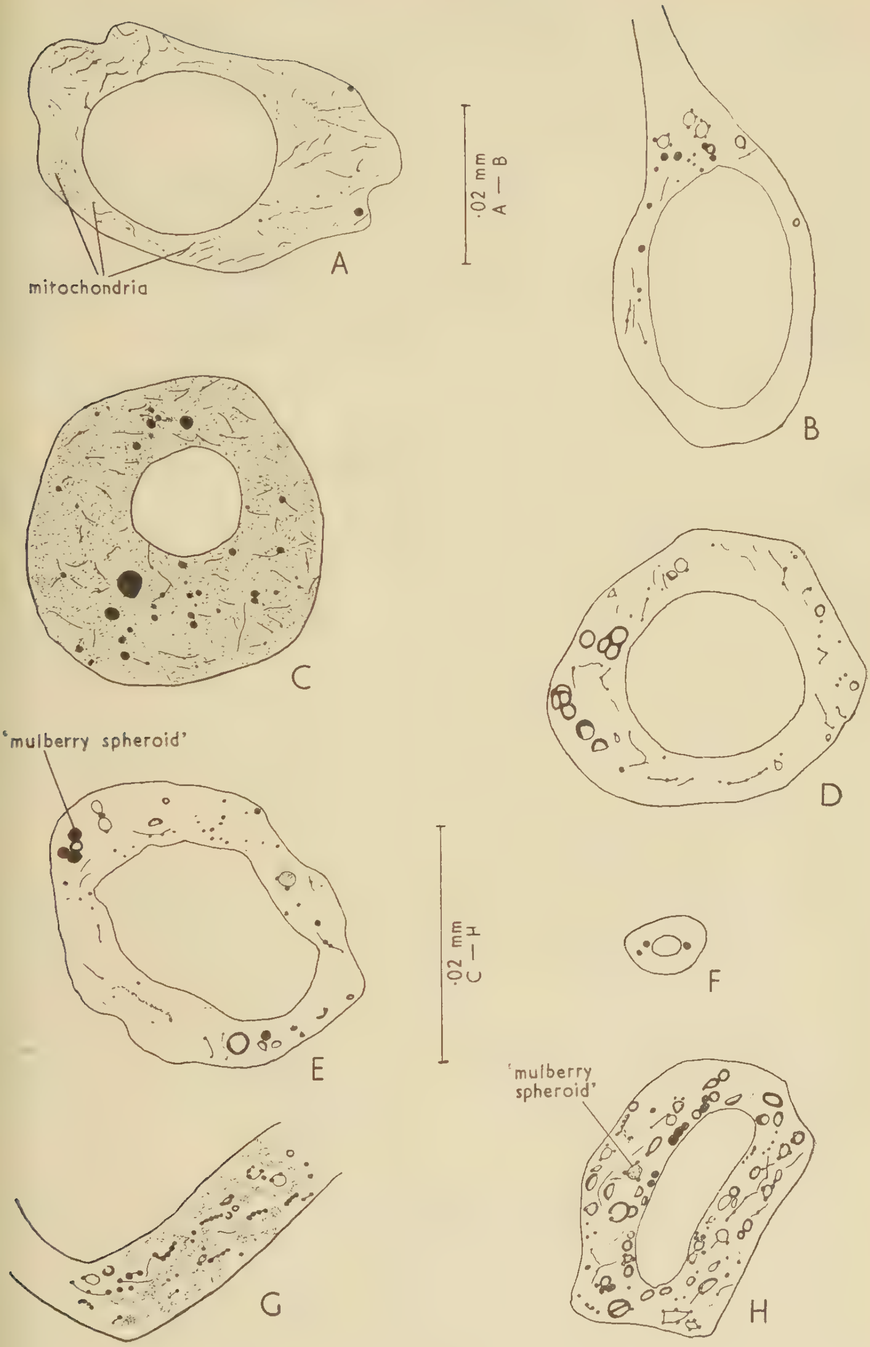

mitochondria
A
·02 mm
A — B
B
C
'mulberry spheroid'
D
E
·02 mm
C — H
F
'mulberry spheroid'
G
H

FIG. 1

Small granules lie on the filamentous mitochondria or at their ends. Thes granules are bigger than the granular mitochondria and are coloured by Suda black (fig. 2, c); it appears that they arise from the mitochondria. These lipi granules represent the intermediate granules of Shafiq (1953) and the smalles homogeneous spheroidal lipochondria of Roque (1954).

The mitochondria are seen by phase-contrast microscopy as greyis granules and filaments scattered throughout the cytoplasm in the livin neurones of *Anadenus*.

Filamentous mitochondria have also been described in the neurones o *Helix* by Thomas (1947) and Roque (1954), but Boyle (1937) and Mouss (1950) described only granular mitochondria in the neurones of *Helix* an *Limnaea* respectively.

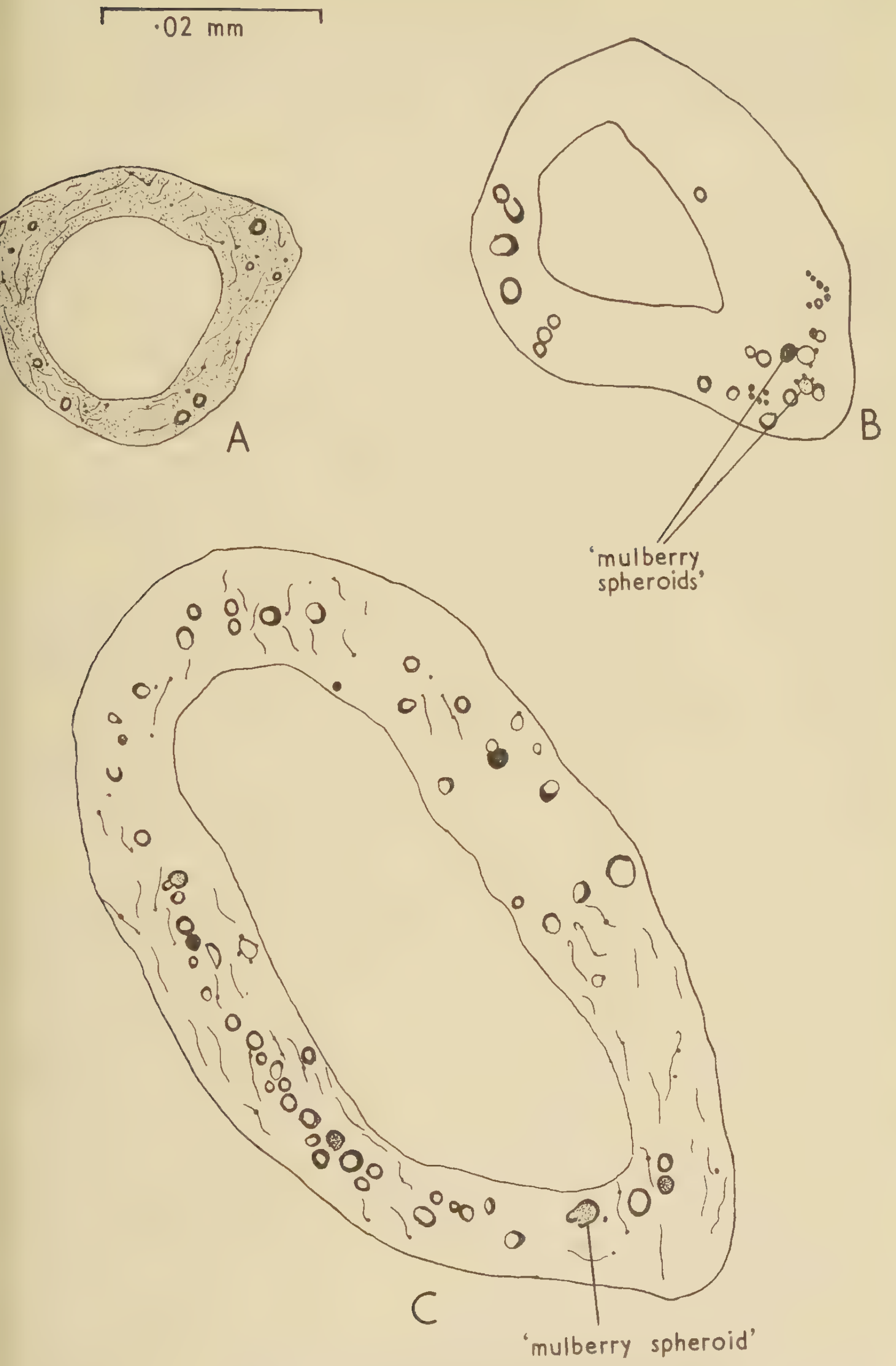
LIPID SPHEROIDS

In view of the existing controversy regarding the nomenclature of the lipi bodies, now often described as lipochondria but originally described as 'Golg elements' or 'Golgi apparatus' in the nerve-cells of various animals, it ha been considered advisable to describe the lipid bodies of the neurones o *Anadenus* and *Euaustenia* as spheroids, in which form they exist in th neurones of these animals.

Phase-contrast study. The living neurones of *Anadenus* have been studied b phase-contrast microscopy. These neurones are rather loosely aggregated i the ganglion, and their cytoplasm does not have such a high consistency as i *Schistocerca* (Malhotra, 1955, 1956). *Anadenus* neurones, therefore, can b easily studied by simply releasing the cover-glass on a small piece of th ganglion lying in a drop of saline solution. No teasing is required; sligh pressure on the coverslip flattens the nerve-cells.

Properly flattened nerve-cells show spheroids of varying sizes. There ar small homogeneous ones, which give a high phase-change and are seen a dark refringent granules. These are identical with the granules that are see lying on the mitochondria or at their ends, as described above. There ar bigger spheroids, which are seen as dark homogeneous bodies. There are als spheroids of intermediate sizes. Most of these show a duplex structure b positive phase-contrast. Each has an outer dark cortex and the inner brigh medulla. The medulla is generally ensheathed by the cortex, but sometime only partially so. In the latter case the cortex appears as a crescent round th spherical medulla.

The medulla of the duplex spheroids present variable phase-changes. Th medullas of a few of them present negligible phase-change, and appear almost colourless; others present various degrees of higher phase-change. Th medulla of a spheroid never shows as much phase-change as the cortex. Th increase in the phase-change of the medulla is due to the gradual condensatio of lipochrome in it. The difference in the phase-change between the lipo-chrome-loaded medulla and the cortex of a duplex spheroid is generally small. Consequently such spheroids may appear as dark, homogeneous bodies.



IG. 2. Neurones of *Anadenus*. Helly / Sudan black, showing the various forms of spheroids bserved, and condensation of lipochrome in figs. B and C. Mitochondria are also seen in figs. A and C.

Lipochrome-loaded spheroids are sometimes slightly distorted and resemble the 'mulberry spheroids' of Thomas (1947).

Sometimes the spheroids lie in separate groups in the living neurones, instead of being scattered at random.

STUDY OF FIXED CELLS

The observations made on the living neurones of *Anadenus* fully confirm the conclusions drawn from the study of fixed preparations. The following description applies to both species.

The spheroids are invariably present in all the nerve-cells of both the species. The size of the nerve-cells varies considerably in each of the two species. In the smallest nerve-cells observed the spheroids are few (fig. 1, F); but in the larger cells their number increases considerably, and they are seen dispersed throughout the cytoplasm. Sometimes in the largest neurones of *Anadenus* they are mostly confined to a region midway between the nuclear and cell membranes (fig. 2, C); sometimes they are confined to the two ends of the cell (fig. 2, B) or to the axon end (fig. 1, B).

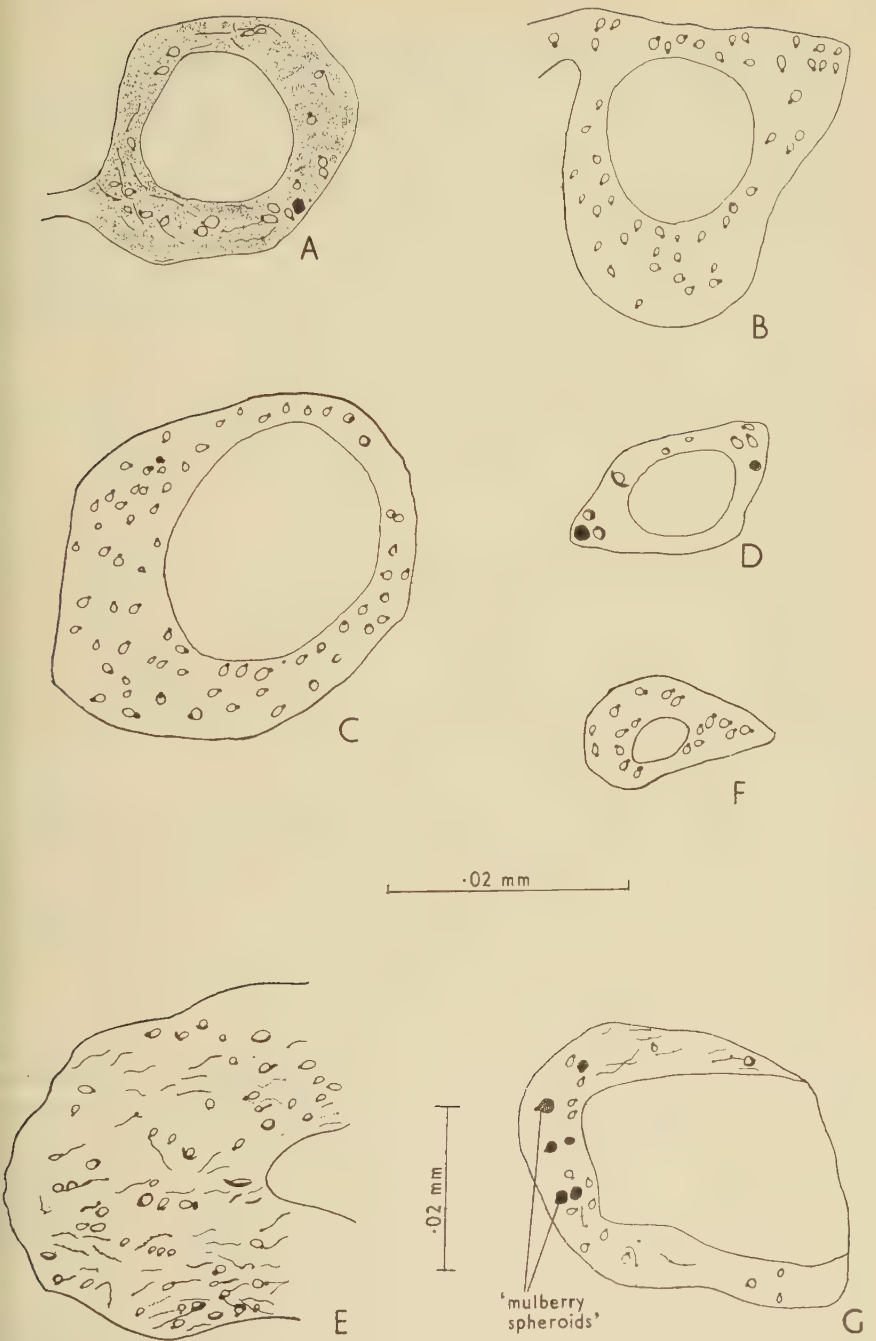
The spheroids vary considerably in size even in the same cell, as has already been described in the phase-contrast study. There appears to be no relation between the size of the spheroids and the size of the cell, for the bigger spheroids are often observed in the smaller cells and conversely. Nevertheless, the number of big spheroids is smaller in the smaller cells.

The spheroids of *Anadenus* neurones are either homogeneous or duplex in structure. In the latter case the cortex may be complete or incomplete, giving the appearances of rings and crescents respectively in optical section; or the cortex may be represented by one or two granules. On the contrary, the spheroids of *Euaustenia* never show a complete cortex.

Helly and Altmann techniques. Helly preparations stained with iron haematoxylin show the spheroids as darkly stained homogeneous bodies of varying size in the neurones of *Anadenus* (fig. 1, A, C). The duplex structure of the spheroids cannot be brought out by this technique. In the neurones of *Euaustenia*, since the chromophil cortex of the spheroid is always incomplete, the medulla can be seen as a distinct, clear vacuole in such preparations (fig. 3, A).

The spheroids are stainable with acid fuchsin (fig. 1, B) when Helly preparations are stained with Masson's tricolour stain. In such preparations the spheroids may appear as homogeneous bodies, or they may show a clear differentiation between cortex and medulla. These preparations of *Anadenus* neurones also show a number of fuchsinophil granules on the surface of a large, pale granule (fig. 1, B).

The granular or crescent-shaped cortical component of the spheroids of the neurones of *Euaustenia* is blackened by Altmann fixation. When unstained Altmann preparations are studied immediately after mounting, the cortical component is seen as dark grey, and the medulla of the spheroids as a clear



IG. 3. Neurones of *Euaustenia*. A, Helly / haematoxylin, showing mitochondria, ordinary pheroids, and 'mulberry spheroid'. B, Altmann unstained. C, Aoyama, showing optimal imregnation. D, Aoyama, showing jet-black 'mulberry spheroids' and excessive deposition of ilver. E, formaldehyde-osmicated for 48 hours, showing formation of artifacts. F and G, Helly / Sudan black.

vacuole (fig. 3, B). When stained with iron haematoxylin it is only the cortic component of the spheroid that is darkly stained.

Golgi techniques. In spite of the established fact that long osmication an treatment with silver nitrate in the Golgi techniques very often introduc serious artifacts in the cell, the picture of the spheroids obtained with thes techniques in *Anadenus* and *Euaustenia* is often the same as with othe methods. In formaldehyde-osmicated preparations of *Anadenus* neurone there are seen small, dark, homogeneous spheroids (fig. 1, D, E). Besides these there are bigger spheroids, which show a duplex structure, that is, each wit an osmiophil cortex and an osmiophobe medulla (fig. 1, E). Sometimes suc spheroids are seen aggregated together in small groups (figs. 1, D; 4, F, G Some of these bigger spheroids show a condensation of a pale yellow materia inside the medulla. Such spheroids appear as black, homogeneous bodies afte prolonged osmication (fig. 1, E). It has also been observed that the fuchsin ophil granules that surround the developing 'mulberry spheroid' are als osmiophil (fig. 1, E).

Besides the above structures, the formaldehyde-osmicated preparations o *Anadenus* also show one or two osmiophil granules or a crescent, closel associated with a rounded osmiophobe medulla (fig. 1, D, E). These forms o the spheroids are the only ones met with in *Euaustenia* (fig. 3, E). Very often the osmiophobe vacuole associated with a crescent is seen collapsed in osmicated neurones of both *Anadenus* (fig. 1, E) and *Euaustenia* (fig. 3, E). Such appearances are obviously artifacts. In extreme cases the vacuole has completely collapsed and does not show at all. It has been observed that osmium is first deposited on the cortical component of the spheroids, then round the spheroids, and, finally, on other structures unrelated to the spheroids. A spheroid round which the osmium has been partially deposited looks like a crescent with the chromophobe substance attached to it (fig. 3, E). Prolonged osmication gives rise to another artifact, namely, the formation of 'rods' without the chromophobe spheres. These rods are obviously formed by the excessive deposition of osmium on the mitochondria. The same remarks apply to silver nitrate techniques.

Aoyama preparations of the neurones of *Anadenus* (fig. 1, G, H) and *Euaustenia* (fig. 3, C, D) neurones generally show the same form of the spheroids as osmium tetroxide preparations, but the duplex spheroids of *Anadenus* neurones mostly become flattened and metallic silver deposits in the form of granules on the argentophil cortex of the spheroids. When there is heavy impregnation it gives results similar to that given by long osmication. In fig. 1, G, H, granulated rods and crescents can be seen, formed by excessive precipitation of silver. But when there is optimal impregnation of silver, the chromophobe substance of the duplex spheroids shows clearly (figs. 1, H; 3, C).

The small, homogeneous spheroids and the cortical component of the duplex spheroid (granule or crescent) appear larger in all dimensions in Golgi preparations than in Sudan black preparations. This is undoubtedly due to the excessive deposition of metallic osmium and silver on the lipid content.

Sudan black. The spheroids in the neurones of *Anadenus* and *Euaustenia* in Sudan black preparations appear essentially in the same forms as in the techniques previously described.

Paraffin sections of *Anadenus* neurones fixed in Helly and coloured with Sudan black show mostly duplex spheroids, each with a thick sudanophil cortex and a lightly staining medulla (fig. 2, A–C). In such preparations spheroids with an incomplete sudanophil cortex investing a rounded chromophobe medulla also appear (fig. 2, B, C). The sudanophil material may be even heaped up on one side of the chromophobe medulla, which shows as a thin sudanophil rim round it (figs. 2, C; 4, B), or it may be restricted to a granule closely associated with a chromophobe medulla (figs. 2, B, C; 4, H).

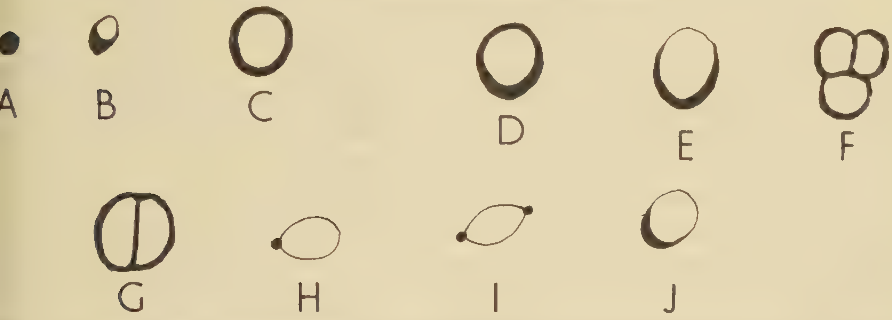


FIG. 4. Diagrammatic representation of the various forms of spheroids observed in different preparations of *Anadenus* and *Euaustenia*.

In the neurones of *Euaustenia* the cortical sudanophil component of the pheroids is mostly restricted to a single granule associated with a chromophobe medulla, but sometimes it may appear in the form of a crescent fig. 3, F, G).

Besides the above forms of spheroids, Sudan black preparations of *Anadenus* nd *Euaustenia* neurones also show small, homogeneous, sudanophil granules cattered throughout the cytoplasm and mostly lying on the mitochondrial hreads (figs. 2, A, C; 3, G). Sudanophil crescents with chromophobe subtance in the concavity of the crescent are also seen.

The cortical component of the duplex spheroids of the neurones of *Anadenus* and *Euaustenia* appears to be lipid, as it colours with Sudan black, reduces osmium and silver, and is darkly stained by haematoxylin fter chrome-osmium and Helly fixation. It is stainable with acid fuchsin Masson's tricolour stain).

This lipid material is variously distributed round the chromophobe subtance of the spheroid (fig. 4), as shown by Baker (1949) in the neurones of the nterior mesenteric ganglion of the mouse.

It is in the chromophobe medulla of the spheroids of the neurones of *Anaenus* and *Euaustenia* that the lipochrome develops, but only few of the pheroids contain the pigment. When the lipochrome first makes its appearnce, it takes up a light stain in Sudan black preparations (figs. 2, C; 3, G) in contrast to the dark cortex. Later, when its amount increases, it colours darkl (fig. 2, C). Sometimes the lipochrome-loaded spheroids are seen as homogeneous bodies in Sudan black preparations (figs. 2, B, C; 3, G). In Golg preparations the lipochrome-loaded spheroids are seen as black homogeneou bodies (figs. 1, E, H; 3, D), and the same is true of Helly-haemotoxylin preparations (fig. 1, C). Sometimes the spheroids which have accumulated lipochrom get so much distorted that the cortex of the spheroid may get torn on one side (fig. 2, C). The 'mulberry spheroids' of *Helix* neurones (Thomas, 1947 are formed in this way.

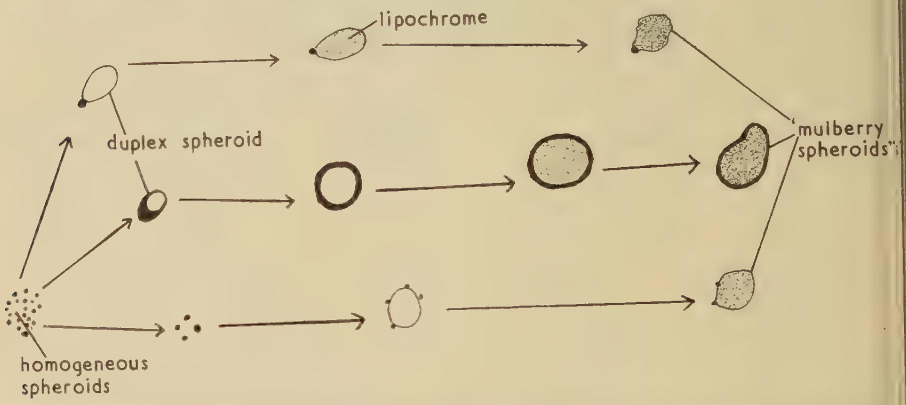


FIG. 5. Schematic illustration of the two methods of formation of 'mulberry spheroids' in *Anadenus* and *Euaustenia*.

Anadenus neurones also reveal the formation of 'mulberry spheroids' of the vertebrate type (Thomas, 1948). In this case three or four small homogeneous lipid spheroids come to lie close together and form 'mulberry spheroids' (figs. 1, B, E, H; 2, C; 5). These small homogeneous spheroids can be compared, in conformity with Thomas (1948), to the Golgi remnants of Hirsch (1939). Fig. 5 is a schematic illustration of the two methods of the formation of 'mulberry spheroids'.

Sudan IV does not colour anything in paraffin sections of neurones fixed in Helly.

DISCUSSION AND CONCLUSIONS

The spheroids of the neurones of the slug *Anadenus* and the snail *Euaustenia* exist either as small and homogeneous or bigger, duplex bodies. The former colour with Sudan black. Most of these homogeneous spheroids lie on the mitochondria. They are bigger than the granular mitochondria and seem to arise from the latter.

The duplex spheroids show an outer chromophil, lipid cortex (sudanophil, osmiophil, and argentophil) and an inner chromophobic medulla. The cortical lipid component is variously distributed round the medulla, as shown by Baker (1949) in the neurones of the anterior mesenteric ganglion of the mouse. In *Anadenus* the lipid component may be in the form of a complete or an incomplete (crescentic) sheath, or it may be restricted to one or two anules in close association with the medulla; but in *Euaustenia* the spheroids ever show a complete cortex.

The duplex spheroids, having a complete lipid sheath round the chromoobe medulla, as are seen in the neurones of *Anadenus*, are identical with the olgi bodies' of Baker (1949) in the nerve-cells of the mouse, and of Maltra (1955 and 1956) in the nerve-cells of *Schistocerca*, and with the 'lipoondria' of Shafiq (1953, 1954) in the nerve-cells of *Locusta* and Roque 954) in the nerve-cells of *Helix*. Further, the spheroids observed in the eurones of *Euaustenia* are certainly identical with the 'binary spheroids' of homas (1947) in the neurones of *Helix* and the 'Golgi bodies' of the present thor (1955, 1956) in the neurones of *Laccotrephes*.

Roque (1954) describes the lipochondria as homogeneous spheroids in the eurones of *Helix*. He interprets the appearance of a cortex or a crescent in the ochondria of living neurones, as seen by phase-contrast microscopy, as tical artifacts, or as artifacts due to incomplete staining with supravital dyes. similar view that the cortex is an optical artifact in the living neurones of *ocusta* studied with phase-contrast, and that it is an artifact due to the deposion of osmium in Golgi preparations, has been put forward by Shafiq (1953 d 1954). But the present author, supporting his phase-contrast observations Sudan black preparations, disagrees with the interpretations of Roque and hafiq. He described the spheroids as genuinely duplex in structure in the eurones of insects (1956). This view of the present author is supported by his udies of the neurones of *Anadenus*.

Moussa (1950) also described duplex Golgi bodies, each consisting of an smiophil and argentophil 'dictyosome' associated with a chromophobe rchoplasm' in the neurones of *Limnaea*. A similar form of the 'Golgi apparus' has also been recorded by Boyle (1937) in the neurones of *Helix*; but oyle describes the chromophobe component of the Golgi dictyosomes as the cretion product of the 'Golgi apparatus'.

Thomas (1948) described 'binary spheroids' in the sympathetic neurones of e mouse, similar to spheroids (Golgi bodies) observed in *Helix* neurones by e same author (1947). From the morphological similarity of the form of the heroids in the neurones of the mouse and *Helix*, Thomas (1948) derived the nclusion that the spheroids of vertebrate and invertebrate nerve-cells are mologous to each other. The latter conclusion has been confirmed by Shafiq d Casselman (1954) and Casselman and Baker (1955). These authors have emically analysed the lipochondria of the neurones of *Locusta* and the rabbit spectively, and have recorded the presence of cerebroside and phospholipid common chemical constituents in the lipochondria of both these animals.

My observations also lead me to the conclusion that the bigger spheroids ow a duplex structure in the neurones of both the gastropod species, *nadenus* and *Euaustenia*, and that the cortical component is the true lipid udanophil, osmiophil, and argentophil) of the spheroids. It is this lipid aterial of the spheroid which represents the Golgi material.

The real form of the lipid material can be studied in Sudan black preparations, as recommended by Baker (1949). It has also been observed, in co formity with Baker (1954), that long osmium and silver techniques introdu artifacts, as the metals are precipitated on the lipid component. If there is optimal impregnation of osmium tetroxide or silver nitrate, the picture of t lipid component seen in the finished preparation is, however, similar to th given by Sudan black. Prolonged impregnations result in the deposition osmium or silver first on the spheroids and finally on other structures u related to the spheroids (Baker, 1954; Malhotra, 1956).

In silver preparations the chromophobe medulla of the spheroids becom swollen, and most of the spheroids are seen as flattened platelets in *Anaden* neurones. Exactly similar artifacts are produced in *Schistocerca* neurones pr pared by Kolatchev's method (Malhotra, 1956).

The medulla of the duplex spheroids does not colour with Sudan blac and it is not blackened by osmium or silver. It has been established by Thom (1947, 1948), Baker (1949), and Malhotra (1955, 1956) that the medulla of t Golgi elements is stainable in life with the basic dye, neutral red. The 'lip chondria' of Shafiq (1953, 1954) and Roque (1954) are also stainable wi neutral red. It is, therefore, concluded from the above facts that the lip material and the bodies that stain with neutral red ('vacuome' of Parat) exi closely associated with each other as duplex spheroids in the neurones molluscs, as in the neurones of *Schistocerca* and *Laccotrephes* (Malhotra, 195 1956). Similarly Roque (1954) in *Helix*, Nath and Gupta (1956) in *An* *denus* and *Euaustenia*, and Nath (1956) in his comprehensive review of th cytology of spermatogenesis, have all described with phase-contrast micro scopy duplex Golgi spheroids, each with a complete or incomplete cortex o the neutral red vacuole.

Young (1932) also described granular, osmiophil, and argentophil Golg bodies and neutral red staining vacuoles as lying close to each other in th neurones of cephalopods.

It also has been observed, in conformity with the findings of Thomas (194 in the neurones of *Helix*, and of Moussa (1950) in those of *Limnaea*, that lipo chrome appears in the chromophobe medulla of the spheroids of the neurone of *Anadenus* and *Euaustenia*. Cain (1948) had demonstrated that the lipo chrome contains carotenoids accumulated in the 'internum' of the spheroid o the neurones of *Limnaea*, *Planorbis*, and *Helix*.

Small homogeneous spheroids, corresponding to the Golgi remnants, als contribute to the formation of 'mulberry spheroids' by their concerted effort in the neurones of *Anadenus*, as in the sympathetic neurones of the mous (Thomas, 1948). Roque (1954) considered that the formation of 'mulberr spheroids' was due to pigment granules becoming attached to the lipochondri in the neurones of *Helix*.

The lipochrome may be regarded as a 'Golgi product', in accordance wit Thomas (1948) and Moussa (1950).

It is a matter of great privilege to me to express my gratitude to Professo

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REFERENCES

AKER, J. R., 1944. Quart. J. micr. Sci., **85**, 1.

—— 1949. Ibid., **90**, 293.

—— 1954. Ibid., **95**, 383.

OYLE, W., 1937. J. Roy. micr. Soc., **57**, 243.

AIN, A. J., 1948. Quart. J. micr. Sci., **89**, 421.

ASSELMAN, W. G. B., and BAKER, J. R., 1955. Ibid., **96**, 49.

ATENBY, J. B., and MOUSSA, T. A. A., 1949. La Cellule, **53**, 15.

IRSCH, G. C., 1939. Quoted by Bourne, 1951. *Cytology and cell physiology*. Oxford U.P., New York.

ALHOTRA, S. K., 1955. Nature, **176**, 886.

—— 1956. Quart. J. micr. Sci., **97**, 177.

OUSSA, T. A. A., 1950. J. Morph., **87**, 27.

ATH, V., 1956. International Rev. of Cytology, **5**, 395.

—— and Gupta, B. L., 1956. Quart. J. micr. Sci., **97**, 369.

OQUE, A. L., 1954. J. Roy. micr. Soc., **74**, 188.

HAFIQ, S. A., 1953. Quart. J. micr. Sci., **94**, 319.

—— 1954. Ibid., **95**, 305.

—— and CASSELMAN, W. G. B., 1954. Ibid., **95**, 315.

HOMAS, O. L., 1947. Ibid., **88**, 445.

—— 1948. Ibid., **89**, 333.

OUNG, J. Z., 1932. Ibid., **75**, 1.

Cyclical Changes in the Distribution of the Testis Lipids of a Teleost Fish, *Esox lucius*

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With two plates (figs. 1 and 2)

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SUMMARY

A study of the cyclical changes in the distribution of testis lipids in the pike reveals a sequence of events comparable with that occurring in seasonal birds. These events include the post-nuptial appearance of cholesterol-positive lipid material which accumulates within the seminiferous lobules, and its subsequent gradual disappearance. A lipid cycle occurs also in the lobule walls, where apparently fibroblasts, in the absence of a true secretory interstitium, become glandular and probably take on an endocrine function. These 'lobule boundary cells' seasonally accumulate cholesterol-positive cytoplasmic lipids which suddenly become depleted at the time of the annual pre-spawning assembly.

The pike differs from wild birds in the 'timing' of such rhythmical activity. In birds, despite a rapid post-nuptial interstitial regeneration, the tubule cholesterol lingers until spermatogenesis begins during the following late winter or spring. In the poikilothermous pike, on the other hand, the next spermatogenesis begins almost mmediately (in June, when the temperature of the water is still rising). It continues without interruption while the length of day and later the temperature decrease, until the testis reaches its maximum size in December. 'Lobule boundary cells' start to become lipoidal in September, at a time of high temperature but decreasing day-length. The cells are fully charged by December. Both tubules and gland-cells now become nactive. Then in April at the spring period of increasing day-length, and immediately after water temperature starts to rise, the boundary cells begin to secrete and the pre-spawning assembly occurs. This is followed by the shedding of spermatozoa later in the month or early in May. The only period of true inactivity is at the height of spermatogenesis during mid-winter and early spring.

INTRODUCTION

THERE exists an extensive literature dealing with the seasonal changes in the gonads of birds, but those of fish are still little understood. In the male bird there has been demonstrated a cyclical regeneration and exhaustion of the cholesterol-positive Leydig cells of the testis interstitium, a regular

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post-spermatogenetic tubule steatogenesis (also involving the production cholesterol), and a post-nuptial rehabilitation of the *tunica albuginea* (Marsha 1949). These events follow an almost invariable time sequence under averag environmental conditions although the rates and events are subject to pr found disruption during periods of climatic fluctuation (Marshall, 1955).

The purpose of the present study was threefold:

1. To determine whether in the seminiferous and endocrine elements of th testis of a fish there occur events comparable with those in birds.
2. To discover whether fish (which also undergo gross seasonal tubu expansion) annually renew their old, stretched, and presumably weal ened *tunica albuginea* as do birds.
3. To correlate, as far as possible, observed events in the testis cycle wit external fluctuations in photoperiodicity and temperature.

MATERIALS AND METHODS

The present study is based on the examination of 71 adult male pike (*Eso lucius* L.) collected at Lake Windermere and nearby Tarn Hows from Octobe 1954 to September 1955, inclusive. The testes were weighed, sliced, an immersed in formaldehyde-calcium. In addition, 42 char (*Salvelinus wil lughbii*) were netted whilst migrating into shallow water to spawn in Octobe and November 1954. Their gonads were removed and preserved for histo logical comparison with those of the pike. The material was treated in thre ways:

Portions of each organ were (1) embedded in wax, sectioned at 6 μ, an stained with iron haematoxylin and orange G for routine examination c spermatogenetic stages; (2) embedded in gelatine, sectioned at 8 μ on th freezing microtome, coloured with Sudan black and stained with haemalum for the investigation of tubule and interstitial lipids; and (3) sections wer subjected to the Schultz test for the detection of cholesterol. All measurement in μ refer to material embedded in wax.

ANATOMY OF THE TESTIS

The testes of the pike are elongated bodies situated below the swim-bladder They extend along the whole length of the abdomen. Seasonal volumetri variation is great. Before the pike begin their spawning run, during January t March inclusive, the gonads are large and often lobulate. The shedding of th spermatozoa, however, results in a striking reduction in volume and weigh (table 1).

The testis differs in internal structure from the characteristic vertebrat pattern of seminiferous tubules and interstitium. A piece of teased testis i seen to consist of a mass of elongated, branching, blindly ending structure applied closely together. Turner (1919) described such structures radiating from a connective tissue core in the perch (*Perca flavescens*) and called them *lobules*. No such core was found in pike, but otherwise the basic structure i imilar. Hann (1927) used the term *tubule* to describe structures in *Cottus airdii* that apparently correspond with the lobules in perch and pike. The *anals* of the stickleback (*Gasterosteus pungitius*) described by van Oordt 1924) are obviously the same structures, and so too are the *crypts* mentioned y Jones (1940) in *Salmo salar*. Since teleosts lack tubules with permanent erminal epithelium like that of tetrapods, the term lobule will be used in this ontribution.

ABLE 1. *Seasonal variations in testicular weight of* Esox lucius. *The figures in rackets refer to specimens taken from Tarn Hows, which, although somewhat smaller than those from Lake Windermere, were histologically similar*

Month	*No. examined*	*Average testis weight (g)*
October	6	66·7
November . . .	10	52·9
December . . .	5	59·7
January	5	57·3
February . . .	1	54·3
March	4 (5)	41·4 (21·3)
April	5 (5)	38·4 (17·3)
May	5 (4)	8·5 (2·7)
June	3 (2)	3·8 (0·8)
July	1	4·4
August	1	6·3
September . . .	6	68·2

The lobule wall in pike is composed of fibrous connective tissue. There are interstitial Leydig cells. During the seasonal cycle, however, most, if not l, of the fibroblasts of the lobule wall become remarkably modified into andular 'lobule boundary cells' which resemble Leydig cells (Marshall and ofts, 1956). As they expand during spermatogenesis the lobules come closer gether. The small inter-lobular spaces become occupied by blood-vessels d lymph spaces. What at first appears to be endocrine interstitial tissue in ld isolated inter-lobular patches is, in fact, the blind ends of sectioned bules. A few are usually seen in any given section.

After spawning has taken place at the end of April, primary germ cells igrate into the collapsed lobules and initiate the new season's spermatonesis. No general agreement has been reached concerning the origin of these lls. Turner (1919) described a 'cord' of germ cells outside the body of the stis of the perch. He claimed that from this the testis is periodically supplied cell-migration into the lobules. These conclusions, however, were based on idence from only one specimen and no mention was made of precisely how e germ cells got from the cord into the testis. Primary germ cells observed us in the inter-lobular tissue of the pike during the post-nuptial period ere similar to those described by Turner, but we found no evidence of an tra-testicular cord. In *Cottus bairdii* some germ cells lie dormant within the sts during maturation and spawning, and these divide during the following summer to provide the next generation (Hann, 1927). There was no evidenc of this in the pike. In *Gambusia affinis* the germ cells are said to develop from inconspicuous cells lying in the interlobular areas of the testis (Geiser, 1922) In the present species we were unable to determine their point of origin.

During summer and early autumn spermatogenesis reaches a peak o activity and the lobules are filled with thin-walled cysts. Spermatogoni divide and give rise to clusters of primary spermatocytes. The cells inside th cysts all divide together so that no cyst contains more than one spermato genetic stage. A cyst with primary spermatocytes contains cells dividin together and will thus come to contain exclusively secondary spermatocytes and next entirely spermatids, and finally spermatozoa. The cyst then rupture and liberates its contents into the lumen of the lobule.

The entire organ is enveloped in a *tunica albuginea* of fibrous connectiv tissue, the thickness of which varies seasonally.

SEASONAL HISTOLOGICAL CHANGES

October. The tunic is about 20 μ thick. The lobules measure from 130 t 180 μ in width and contain cysts of developing primary and secondar spermatocytes and spermatids. Most peripheral cysts are composed c spermatocytes, whereas the more central areas are occupied chiefly but nc exclusively by free spermatozoa (fig. 1, A). A few isolated, free spermatogoni persist in some lobules.

Surrounding each lobule, and incorporated in its substance, is a ring c modified fibroblasts one cell thick. Each individual 'lobule boundary cel contains numerous cytoplasmic lipid droplets (fig. 1, B). These dissolve ou during wax-embedding and leave a series of vacuoles with the appearance c a cytoplasmic reticulum (fig. 1, C). The average size of the nucleus is 5 × 2·5 μ There is no lipid within the lobules. Many of the lipid droplets of the boun dary cells are cholesterol-positive.

November. The average width of the lobule is 180 μ and the tunic is 20 thick. Cysts of all spermatogenetic stages except spermatogonia still occur bu in smaller numbers than in October. The peripheral zone of developing cyst has disappeared. All lobules contain large masses of free spermatozoa.

The cytoplasmic lipids of the lobule boundary cells have increased so tha in many of the cells individual droplets are no longer distinguishable. In suc cells the densely sudanophil cytoplasm resembles that of the Leydig cells c

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FIG. 1 (plate). A, testis in October showing lobules becoming swollen with cysts of spe matocytes, spermatids, and free spermatozoa. The spermatozoa form darkly staining mass in the lobule lumina. Formaldehyde-calcium fixation; 6 μ paraffin section stained with irc haematoxylin and orange G.

B, testis in March. The lobules are full of spermatozoa and the boundary cells of the lobu wall are charged with cholesterol-positive lipids. Formaldehyde-calcium fixation; 8 μ gelati section coloured with Sudan black and haemalum.

C, part of the lobule wall showing lobule boundary cells from which lipids have bee dissolved, leaving a series of small vacuoles in the cytoplasm. Technique as in A.

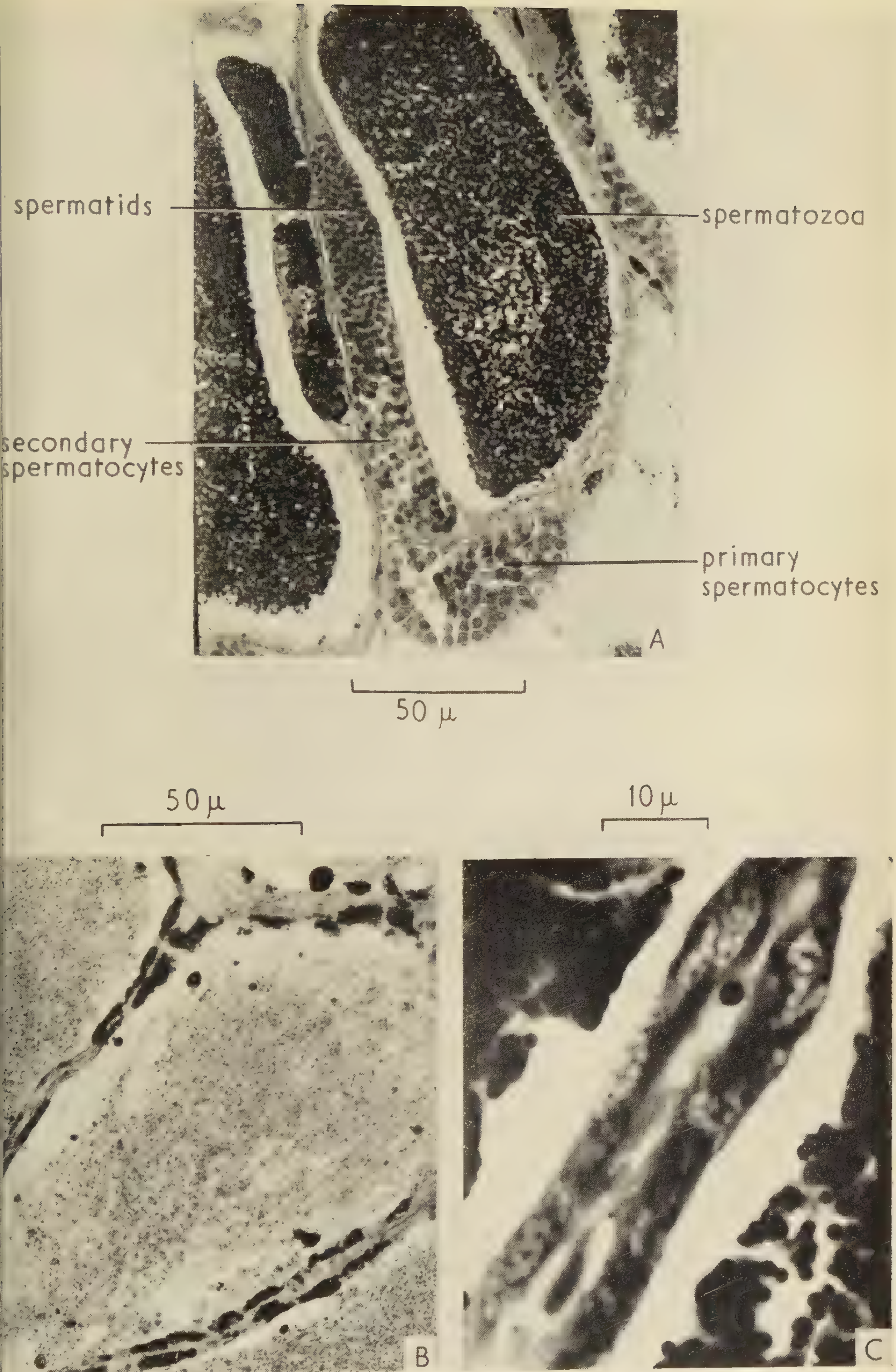

FIG. 1

B. LOFTS *and* A. J. MARSHALL

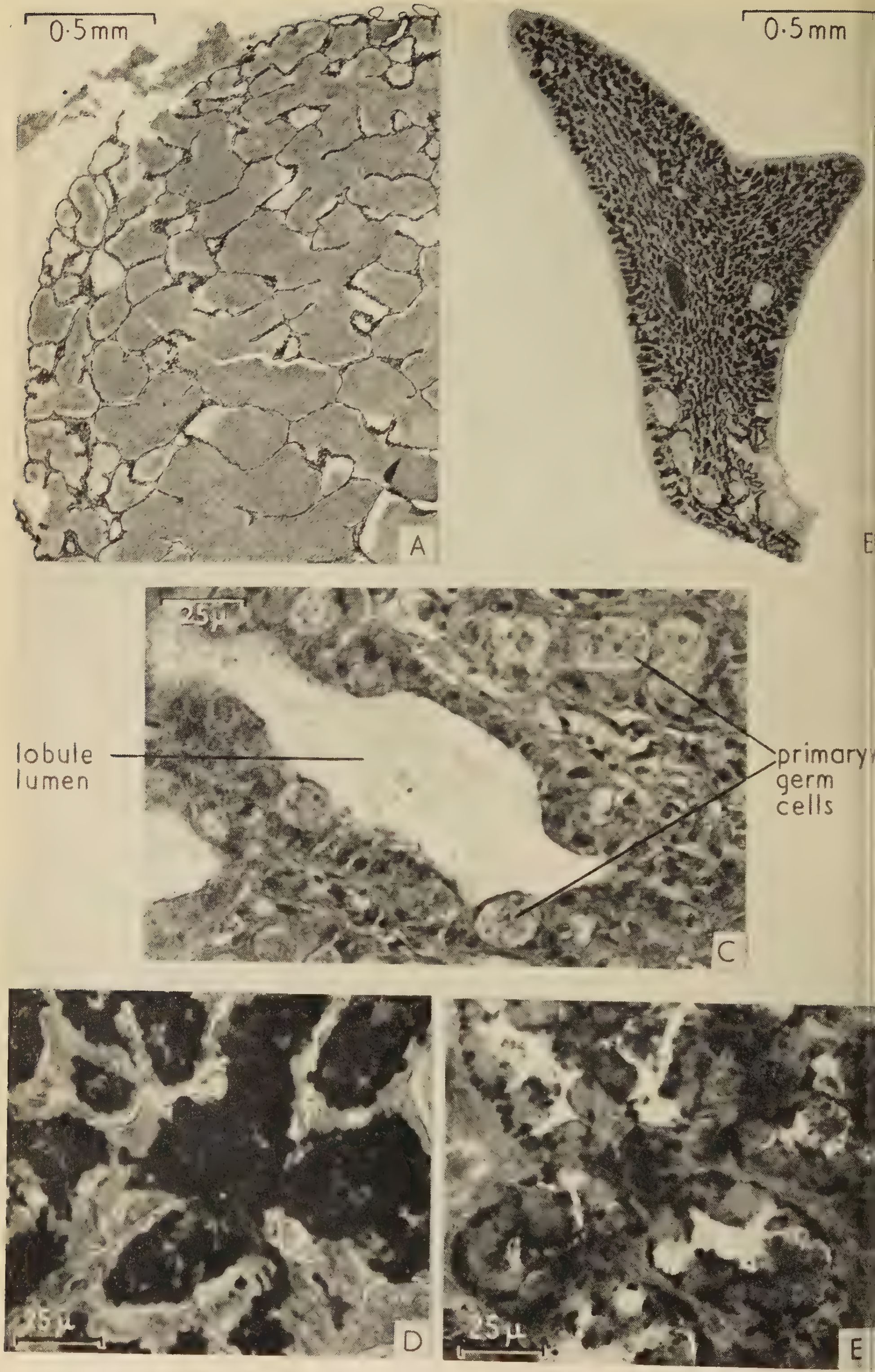

FIG. 2

B. LOFTS *and* A. J. MARSHALL

birds in winter and early spring. This peri-lobular lipid is still cholesterol-positive.

December. Lobule width averages 190 μ and the tunic is 15 μ thick. The cysts are gradually reduced in number. The few remaining contain secondary spermatocytes and spermatids. The lobules have become distended with spermatozoa and are tightly packed together within the testis tunic. There is no change in the lipid or cholesterol content of the boundary cells.

January. The average lobule width is 150 μ. The testis coat is now stretched to a thickness of only 12 μ. All cysts have matured and discharged and the lobules are packed exclusively with mature spermatozoa (fig. 2, A). Lobule boundary cells are still densely lipoidal and cholesterol-positive.

February. The lobule diameter averages 180 μ but there is otherwise no change from January.

March. As in January.

April. Lobules still remain identical in size and appearance with those of January, but the cholesterol-positive boundary cells appear to be discharging their cytoplasmic lipids and cholesterol. Individual lipid globules can be again distinguished. Spermatozoa are discharged at the end of April or the beginning of May.

May. With the shedding of spermatozoa the average lobule is sharply reduced from 180 μ to a width of only 40 μ. The testis tunic has become weakened and appears to be disintegrating. Beneath the old tunic an upsurge of fibroblasts is forming a new one.

The size of the whole testis is sharply decreased (fig. 2, B). There is at the same time an increase in the true volume of inter-lobular tissue. In it occur large primary germ cells; these begin also to appear within the lobules (fig. 2, C). They seem, in fact, to migrate between the cells of the lobule wall into the lumina of the lobules. Such migratory germ cells are about 15 μ in diameter and contain a nucleus 10 μ in diameter. They are probably the same as those described by Turner (1919) and Essenburg (1923). Geiser (1922) and van Oordt (1925) used the term primary spermatogonia for similar cells in *Gambusia affinis* and *Xiphorphorus helleri* respectively.

The lobule wall is now composed of unmodified and lipid-free boundary cells (presumably of a new generation), but the lumen is full of a dense, amorphous mass of densely sudanophil material (fig. 2, D) such as occurs in

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FIG. 2 (plate). A, testis in March (immediately before spawning) showing lobules at maximum size. Lobule boundary cells are heavily lipoidal but lobules are lipid-free. Formaldehyde-calcium fixation; 8 μ gelatine section stained with Sudan black and haemalum.

B, testis in May (immediately after spawning), showing great decrease in size and lobule steatogenesis. Technique as in A.

C, primary germ-cells migrating into the lobule lumen of a 'spent' testis in May. Formaldehyde-calcium fixation; 6 μ paraffin section stained with iron haematoxylin and orange G.

D, showing the dense cholesterol-positive lipid material in lobule lumina of post-nuptial testis in May. Technique as in A.

E, testis in June showing partial clearing of intra-lobular lipids. Spermatogonia are beginning to appear. Technique as in A.

birds at a comparable stage of the sexual cycle (Marshall, 1955). This is cholesterol-positive. Some individuals show the first stages of post-nuptial lobule clearance in that the central areas of their lumina are already free of lipids.

June. The lobules average 50 μ in diameter and the testis tunic is 10 μ thick. The old weakened tunic has disappeared and has been replaced by the proliferation of new fibroblasts from within. Intralobular lipid clearance has continued. Many of the lobules now have their central regions free of lipid (fig. 2, E). Primary germ cells have given rise to spermatogonia. These line the lobules but have not yet multiplied and formed cysts. The fibroblasts surrounding the lobules remain unmodified and lipid-free, having spindle-shaped nuclei measuring 8 to 10 μ in length. Cholesterol remains present in the lobule lipids but none has yet arisen in the potentially glandular boundary cells.

July. The lobules average 50 μ in diameter and the testis tunic is 30 μ thick. Little lipid now remains in the lobules and that which persists is wedged between germ cells. Spermatogenesis is advancing: cysts of primary spermatocytes have arisen. There is still no evidence of glandular boundary cells but an appreciable increase in the number of blood-vessels in the interlobular tissue has occurred. Weak indications of cholesterol still persist in some lobules.

August. The lobules have swollen to a diameter of 120 μ and the testis tunic has stretched to 20 μ in diameter. The lobules are now almost cleared of lipid. The fibroblasts in the lobule borders still remain unmodified and in the July condition. Spermatogenesis, however, is proceeding actively. Every lobule is filled with cysts of dividing germ cells with primary spermatocytes most abundant, although secondary spermatocytes and spermatids are also present. In a few lobules free spermatozoa have appeared. The last traces of cholesterol have disappeared from within the lobules but none has yet arisen in their walls.

September. The average lobule diameter is still 120 μ and the testis tunic has a width of 20 μ. Most lobules now contain at least some spermatozoa. Cysts of primary and secondary spermatocytes and spermatids are numerous: few spermatogonia remain. The fibroblasts of the lobule wall have started to become lipoidal. Individual cytoplasmic lipid droplets are cholesterol-positive.

THE SEXUAL CYCLE AND THE ENVIRONMENT

The internal events described above fall into the following phases:

Phase I. May to August (*period of rehabilitation*)

After the shedding of spermatozoa the remaining lobule-elements undergo steatogenesis; the lumina become filled with cholesterol-positive post-nuptial lipids. This is part of the internal rhythm of the testis. Such lipids now gradually disappear. Meanwhile primary germ cells appear and multiply apidly to produce spermatogonia which in turn divide and form cysts of rimary spermatocytes. The lobule wall consists of unmodified fibroblasts, one of which has begun a seasonal accumulation of lipid. The shrivelled estis tunic is disintegrating. Beneath the old coat new fibroblasts are building p a new tunic.

These rehabilitative processes begin while day-length is increasing and the emperature of the water is rising. On 2 May the temperature at the surface as 9·35° C and this steadily increased to 20·25° C by 31 August. At greater epths the temperature is lower but nevertheless steadily rising (fig. 3). On

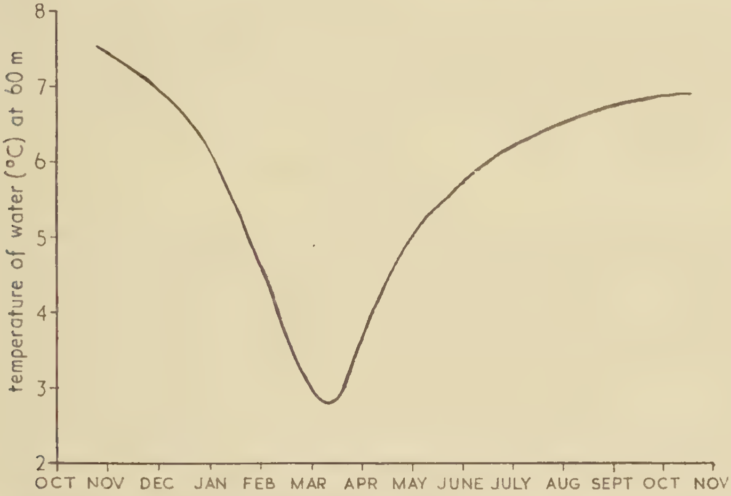

FIG. 3. Seasonal variations in the temperature of the water of Lake Windermere at 60 m.

May day-length is 15 h 5 min and increasing by 4 min a day. These increents extend day-length to 17 h 9 min by 21 June. Day-length then begins decrease and by 31 August it is reduced to 13 h 5 min. Spermatogenesis ontinues unabated during this period of shorter days.

hase II. September to December (period of maturation)

The last traces of post-nuptial lobule lipids disappear and the cytoplasm of e lobule boundary-cells now accumulates cholesterol-positive lipids. There a gradual maturation of the cysts into spermatozoa which are discharged to the lobule lumina. The previous season's testis tunic has disintegrated.

During this maturation water temperature starts falling but on 31 Decemer it is still about 6·75° C. Day-length is also declining and reaches a inimum of 7 h 22 min at the winter solstice (21 December). Throughout eptember the average daily decrease is 4 min. After 21 December ay-length begins to increase but during the rest of December the daily crements are only about 30 sec per day.

Phase III. January to March (period of inactivity)

The boundary-cell cytoplasm is now heavily charged with cholestero positive lipids. Lobules are at their maximum size and are densely packe with free spermatozoa. This stretches the testis tunic to its minimum thicl ness. Meanwhile, the temperature of the water is gradually decreasing an day-length increasing.

Phase IV. April (period of reproduction)

For a while the lobules remain unchanged but the boundary cells begin t discharge their lipids and cholesterol. Spermatozoa are shed at the end c April and early in May. These culminating events occur at a period of risin temperature. Such an increase begins about the third week of March and b the end of April has increased to 8·9° C (fig. 3). On 1 April the day-length i 13 h 1 min and increasing by 5 min a day.

DISCUSSION

In the testis of the pike there occur cyclical events not unlike those c seasonal birds. These involve a post-nuptial steatogenesis of the lobules an a subsequent gradual expenditure of recently arisen cholesterol. In bird however, no more than primary spermatocytes are formed while tubule lipid remain. The last traces of tubule lipids disappear before, or with, the mai upsurge of the next season's spermatogenesis, whereas in the pike, as in *Viper* *berus* and *Rana temporaria* (Marshall, unpublished), spermatogenesis is we under way whilst appreciable quantities of lobule or tubule lipid still remain

The cycle of the boundary cells is essentially similar to that of the inten stitial cycle in birds. The unmodified fibroblasts which arise around the 'sperm lobules grow and accumulate cholesterol-positive lipids in their cytoplasm This occurs also in the teleosts *Salvelinus* and *Labeo* (Marshall and Lofts 1956).

Two distinct endocrine arrangements, therefore, occur in the testes c teleost fish. One arrangement is here shown in the pike and the char: thes lack a true interstitium. The second arrangement conforms to the typica vertebrate pattern (of Leydig cells) and occurs in the stickleback (*Gaster* *osteus*), the sprat (*Clupea*), and many other species of teleosts and also th elasmobranchs *Scylliorhinus* and *Chimaera*. A boundary-cell cycle occurs als in certain urodeles. Miller and Robbins (1954) described in *Tarich* (= *Triturus*) *tortosa* large peri-tubular connective tissue cells with an osmio phil cytoplasm with an apparently glandular function.

In the pike, as in birds, a new testis wall is formed after each breedin season.

A consideration of seasonal fluctuations in day-length and water tempera ture shows that rehabilitative changes, beginning immediately after th discharge of spermatozoa in late April and early May, occur whilst bot day-length and temperature are increasing. Maturation of germ cells (i.e. th transformation of primary germ cells to spermatozoa) occurs from June to December. Thus, decreasing day-lengths after the summer solstice do not inhibit generative processes. By the winter solstice, and at a period when the surface is sometimes covered with ice, the lobules appear to be 'ripe'. They are now distended with spermatozoa and the lobule boundary cells have become heavily charged with cholesterol-positive lipids which are, however, still unexpended. The impression is gained that, whatever the subsequent function of such a cholesterol reservoir, it is not primarily concerned with manufacture of hormone material concerned with spermatogenesis.

During the coldest period of the year, and despite increasing day-length, there ensues a period of inactivity. This lasts until April. Later that month, with the temperature of the water elevated to 8·9° C, the fish undergo an annual pre-spawning assembly and, as their boundary cells suddenly become denuded of cholesterol (probably signifying the manufacture and discharge of sex hormone), spermatozoa are shed.

In the absence of experimental data we cannot evaluate fully the relative importance of temperature and day-length in the control of the events outlined above. Fabricius (1950) has suggested that the spring increase in day-length and light intensity are the external stimuli critical in the pike. That day-length can indeed affect the reproductive cycle of fish has been demonstrated by Hoover and Hubbard (1937), who subjected brook trout (*Salvelinus fontinalis*) to a gradual daily increase and then decrease in illumination, and observed the production of ripe eggs and mature spermatozoa several months earlier than usual. Light may possibly be an effective stimulus when the pike have already assembled in shallow water, but it seems unlikely that it initiates the neuro-hormonal events that lead to such assembly from the main body of the lake. Light intensity is greatly reduced in deep water, and furthermore, assembly occurs in many areas when the water is still covered by ice (Fabricius, 1950; Moen and Lindquist, 1954). Carbine (1941) recorded 18 inches of ice on Haughton Lake when the pike showed the first signs of assembling to spawn.

Increase in water temperature, on the other hand, has been shown to accelerate spermatogenesis in a number of teleosts (Bullough, 1939; Burger, 1939; Matthews, 1939). Individuals of the three-spined stickleback (*Gasterosteus aculeatus*) from different habitats showed a variation in spermatogenetic rates apparently owing to the variation in the rates of temperature reduction in autumn (Craig-Bennett, 1930). When such reduction was rapid, the rate of spermatogenesis was delayed and maturation continued into the breeding season. When slow, spermatogenesis was completed two to three months before the breeding season as in the pike.

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REFERENCES

BULLOUGH, W. S., 1939. Proc. Zool. Soc. Lond., **109**, 79.
BURGER, J. W., 1939. Biol. Bull., **77**, 96.
CARBINE, W. F., 1941. Trans. Amer. Fish. Soc., **71**, 149.
CRAIG-BENNETT, A., 1930. Phil. Trans. Roy. Soc. Lond., B, **219**, 197.
ESSENBURG, J., 1923. Biol. Bull., **45**, 46.
FABRICIUS, E., 1950. Institute of Fresh-Water Research, Drottningholm, Report No. 31.
GEISER, S. W., 1922. Anat. Rec., **23**, 104.
HANN, W. H., 1927. J. Morph., **43**, 427.
HOOVER, E., and HUBBARD, H., 1937. *Copeia*, 206.
JONES, J. W., 1940. Proc. Roy. Soc. Lond., B, **128**, 499.
MARSHALL, A. J., 1949. Quart. J. micr. Sci., **90**, 265.
—— 1955. Mem. Soc. Endocrinol. No. 4, 75.
—— and LOFTS, B., 1956. Nature, **177**, 704.
MATTHEWS, S. A., 1939. Biol. Bull., **77**, 92.
MILLER, M. R., and ROBBINS, M. E., 1954. J. exp. Biol., **125**, 415.
MOEN, T., and LINDQUIST, M., 1954. Prog. Fish. Cult., **14**, 43.
OORDT, G. J. VAN, 1924. Arch. mikr. Anat., **102**, 379.
—— 1925. J. exp. Biol., **3**, 43.
TURNER, C. L., 1919. J. Morph., **32**, 881.

Seasonal Lipid Changes in the Sexual Elements of a Male Snake, *Vipera berus*

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With two plates (figs. 1 and 2)

SUMMARY

In the seminiferous tubules and interstitium of the viper there occur cyclical
anges basically similar to those in birds and the pike. These involve the meta-
orphosis of unshed germinal material into cholesterol-positive lipids, the rehabilita-
n of the interstitium after the seasonal depletion of its cytoplasmic lipids and
olesterol shortly after the spring emergence from hibernation, and the completion
spermatogenesis after basking in the sun. The viper differs from birds in that its
bule lipids contain much less cholesterol; also, the subsequent spermatogenesis
gins almost immediately (as in poikilothermous pike and frog), and proceeds to the
ermatid stage in the presence of such lipids. Further, although in some individuals
w Leydig cells rapidly mature, this resurgence is delayed in others. Thus, although
e succeeding spermatogenesis always begins in still-lipoidal tubules, it may do so
the presence of a largely non-lipoidal interstitium. Mitoses were observed in mature
ydig cells. Fibroblasts occasionally alter to take on an apparently glandular function.

By July most animals had regained a heavy lipoidal and cholesterol-positive
terstitium, lost most of their tubule lipids, and possessed either secondary spermato-
tes or, sometimes, spermatids. This internal activity is probably partly responsible
r autumnal sexual behaviour. Factors additional to temperature control spermato-
nesis, for only a few spermatozoa are formed by the time of hibernation in October.
o pronounced retrogressive changes occur during hibernation: the cycle merely
lts, and begins without further delay on emergence. Thus spermatogenesis may be
its peak by the end of March.

The expanded, glandular 'sexual segment' of the male renal tubule also exhibits
clical changes involving the production and expenditure of cholesterol-positive
ids, but such changes take place also in narrow parts of the tubule and, further, in
e kidney of the female.

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INTRODUCTION

AT the conclusion of spermatogenesis in birds, the seminiferous tubules undergo a metamorphosis involving the sudden breakdown of unused
rminal material and the genesis of a large quantity of cholesterol-positive

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lipids. At about the same time a new generation of Leydig cells arise in exhausted interstitium (Marshall, 1949). The tubule lipids and contain cholesterol, the function of which are unknown, disappear at rates vary between species and with the environmental conditions to which they subjected (Marshall, 1955; Serventy and Marshall, 1956). Although a f primary spermatocytes may arise in the presence of such lipids, the l remaining traces of fat always disappear at the true onset of the succeed spermatogenesis.

Such a complete post-nuptial tubule metamorphosis has not been shown mammals except after hypophysectomy (Coombs and Marshall, 1956), bu is a regular part of the cycle of certain teleost fishes (Marshall and Lofts, 195 The present study was designed to determine whether comparable eve occur naturally in the tubules and Leydig cells of Reptilia and if, in additi cyclical lipid changes take place in the supposedly sexual segment of kidney.

MATERIAL AND METHODS

Vipera b. berus (L.), the common, northern or European viper or adder widely though discontinuously distributed throughout Europe, includi Britain. It extends also across Asia as far as China and Sakhalien (Smi 1951). In Britain hibernation generally begins in October and ends February or early in March, when groups of emergent vipers are sometim found coiled together in the sun. Later, males face each other and perform harmless writhing 'dance' which may be of territorial significance. Th dances often occur near a female. Afterwards the male displays directly t female and copulation occurs in April or May. Like many species of bi (Morley, 1943; Marshall, 1951), the viper undergoes autumn sexual activ as well.

The present study is based on 55 adult males (minimum length 480 m 3 juvenile males (average length 369 mm), and odd adult females collect during two seasons at Carradale, Scotland (lat. 55.36 N), and Doncast England (lat. 53.31 N), in addition to 4 adult males and 5 adult fema experimentally hibernated in London during the winter. The hibernat animals were enclosed on 20 September in a dark but ventilated vivariu measuring 5 by 3 by 2 feet and left in a semi-open situation exposed normal temperature fluctuations but not to rain, wind, or direct light. Th were killed on 30 January.

The vipers were caught by means of artery forceps and killed by decapi tion. Fragments of testis, kidney, fat body, and epididymis were removed a pieces of each tissue fixed in formaldehyde-calcium (FC) or Bouin's flu The FC material was embedded in gelatine, sectioned at 10 μ on the freezi microtome, coloured with Scharlach R for the demonstration of lipids, a counterstained with Ehrlich's haematoxylin. Sections were submitted also the Schultz test for cholesterol, with a section of the adrenal as a contr (Subperitoneal depot fat was never positive to the Schultz test.) The Bo material was embedded in paraffin wax, sectioned at 7 μ, and stained with iron haematoxylin for the examination of spermatogenetic stages. All measurements in microns refer to paraffin sections.

HISTOLOGY OF TESTIS AND KIDNEY

The testis falls into the typical vertebrate pattern of seminiferous tubules and prominent glandular interstitial Leydig cells (fig. 1, A, B). The presumed sexual segment of the kidney (Gampert, 1866; Regaud and Policard, 1903) may constitute up to one-half of the total organ in some males. Viewed in cross-section, the male kidney is seen to contain two distinct tubular components. Each appears to represent a different part of the same renal tubule: the narrow (about 30 μ) renal element is proximal to the glomerulus; the wider (up to 180 μ), obviously glandular, 'sexual' structure (fig. 2, C) arises sub-terminally in sharp transformation. This segment is composed of columnar cells (fig. 2, D) in which, apparently, secretion granules have been demonstrated. It is said to be most active during the spring maturation of spermatozoa, when supposed secretory products are discharged into the lumen (Volsoe, 1944). A profuse capillary system ramifies in the interstices between the tubules.

SEASONAL DISTRIBUTION OF TESTIS LIPIDS

31 March (*6 specimens*)

Seminiferous tubules. These were up to 260 μ in diameter, according to the individual. Spermatozoa were already present in great numbers and mitotic activity occurred throughout the germinal epithelium. Even basal spermatogonia were in division. In the more sexually advanced males spermatids occurred in the sub-peripheral row of cells. A dense concentration of lipid material had arisen near the tubule peripheries (fig. 1, A). A less profuse accumulation occurred in the central areas, which were often still without germ-cells. Such tubule lipid was generally weakly positive for cholesterol but was occasionally negative. In wax sections large vacuoles, from which lipids had been removed, were obvious in the spermatogonia. The basal lipids, therefore, did not come principally from the Sertoli cells nearby.

Leydig cells. These were arranged singly (as viewed in cross-section) or in tracts of up to 20. Individually they measured generally some 12 μ wide with a nucleus about 6 μ in diameter, but in some testes they were as big as 14 μ (nucleus 8 μ). The cytoplasm of many was densely sudanophil, but in some the remaining lipids were restricted to large, separate globules. In others, interstitial lipid had almost disappeared (fig. 1, A)—an index of the seasonal expenditure of the contained cholesterol. All interstitial lipid was heavily cholesterol-positive. In addition there had arisen numbers of small Leydig cells which measured about 8 μ in diameter (nucleus 4 μ). These were non-lipoidal.

Epididymides. These contained massed spermatozoa in some animals but were still almost empty in others.

16 April (8 specimens falling into two categories)

1. *Less advanced group*

Seminiferous tubules. These were up to 300 μ in diameter and contained great numbers of spermatozoa. Spermatogenesis was still proceeding at the tubule peripheries. At the same time a dense aggregation of lipid material was retained at the peripheries and some still occupied the central areas, which were now devoid of germ-cells. The peripheral lipids were sometimes Schultz-negative; the central material was weakly positive.

Leydig cells. As in March, except that in general a greater seasonal depletion had occurred, even though the cells remained of the same size. A wide range of variation in lipid content occurred in the same organ. The remaining lipids were always heavily positive for cholesterol. Considerable numbers of small non-lipoidal cells also occurred.

Epididymides. All contained abundant spermatozoa. Some, however, appeared to have partly shed their contents.

2. *More advanced group*

Seminiferous tubules. Post-spermatogenetic collapse had begun and the diameter was reduced to about 160 μ. The centre of each tubule was occupied by a dense aggregation of lipid (fig. 1, B) that was weakly positive for cholesterol. An irregular peripheral zone of lipid material was still retained and it only sometimes gave a positive Schultz reaction. The epithelium was now confined to about five layers, only a few of which were in division. The current spermatogenetic rhythm had almost ended.

Leydig cells. Many groups of heavily lipoidal and cholesterol-positive interstitial cells were present (fig. 1, B). Some vacuolated, non-lipoidal cells still remained. Greater numbers of smaller cells had arisen but these as yet lacked cytoplasmic lipids.

Epididymides. These contained relatively few spermatozoa but, in addition, some lymphocytes. Those of some vipers were now almost empty again (e.g. the animal depicted in fig. 1, B).

FIG. 1 (plate). A, 31 March. Testis approaching height of spermatogenesis. Huge numbers of spermatozoa (some indicated by top ring) are generally masked by tubule lipids that are weakly positive for cholesterol. Interstitial Leydig cells (bottom ring) have been depleted of lipids and cholesterol (compare B). (FC, 10 μ, Scharlach R.)

B, 16 April. Testis showing heavy tubule steatogenesis and pronounced interstitial rehabilitation at the conclusion of spermatogenesis. (Technique as in A.)

C, 18 May. Testis showing cessation of spermatogenetic activity, despite interstitial rehabilitation. A few spermatozoa remain unshed. The clear tubule lumina and sub-peripheral vacuoles held lipids in the living reptile. (Bouin, 7 μ, iron haematoxylin.)

D, 18 May. Testis showing tubule clearance proceeding in an animal with largely exhausted interstitium (ringed). (Technique as in A.)

E, 31 May. Testis showing renewal of spermatogenesis with the production of secondary spermatocytes. An interstitial mitosis is ringed. (Technique as in C.)

F, 24 July. Testis with tubules almost cleared of post-nuptial lipids and now full of developing germ-cells alongside a fully regenerated interstitium. (Technique as in A.)

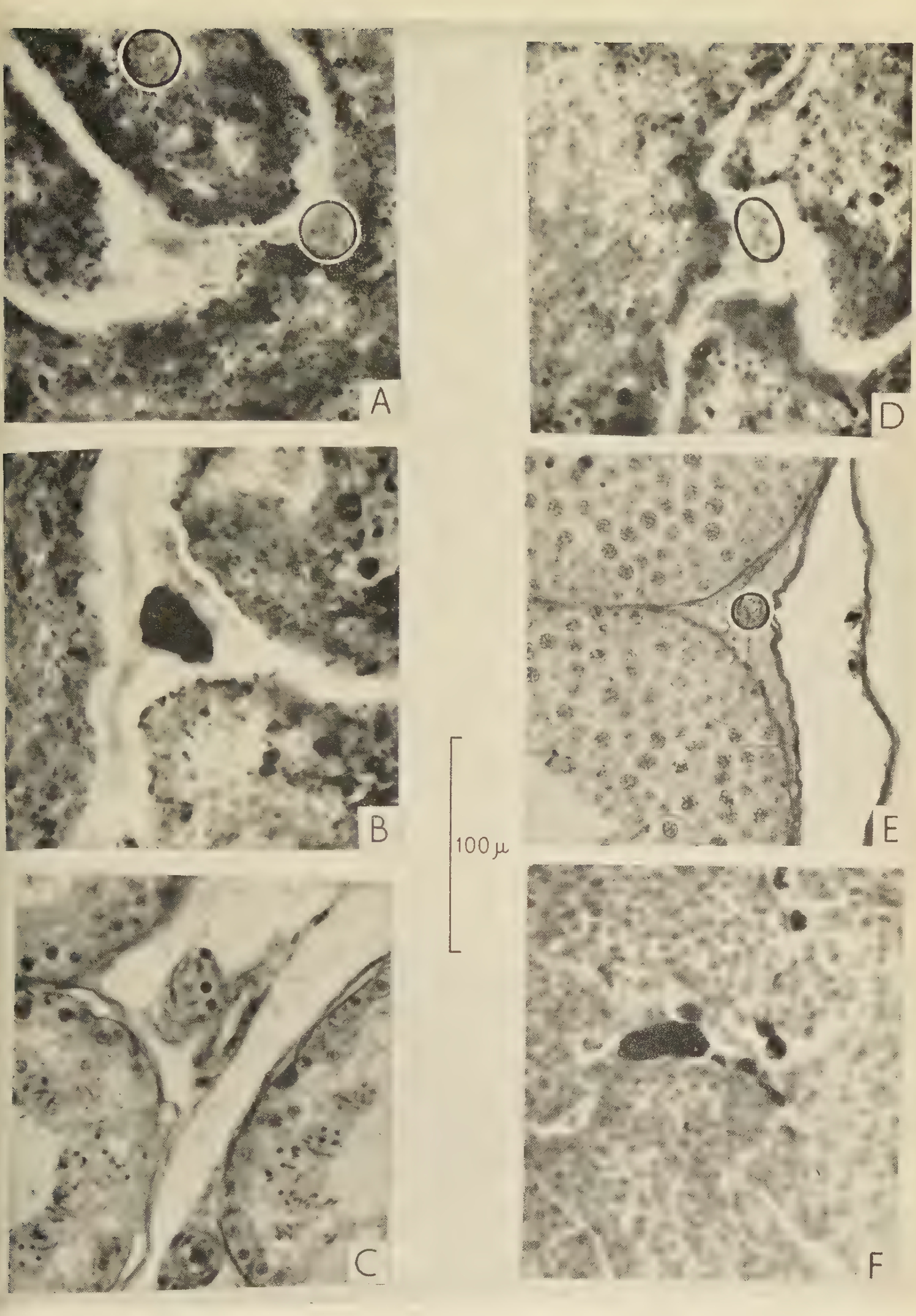

Fig. 1

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8–22 May (9 specimens falling into three categories)

1. Similar to less advanced group (category 1) of April except that two nimals had an apparently completely exhausted interstitium in which it was mpossible to demonstrate cholesterol.

2. Similar to the more advanced group (category 2) of April, except that, n addition, some spindle-shaped fibroblasts of the interstitium had become holesterol-positive and so would apparently become part of the endocrine omplement of the organ. Some exhausted Leydig cells now had pycnotic uclei.

3. Most advanced category, as follows:

Seminiferous tubules. These were reduced to a width of 160 to 180 μ ccording to individual animals. Few spermatozoa remained except for rregular clusters here and there. Aggregations of spermatids were still present ut mitotic activity had stopped (fig. 1, c). In two animals not a single mitosis vas observed among the spermatogonia. All tubules still retained varying uantities of lipid material in the peripheral regions and elsewhere.

Leydig cells. Clusters of mature, heavily lipoidal cells occurred. It was mpossible to determine whether some of these constitute unexpended lements of the former sexual rhythm, or whether, more probably, all had uickly matured from the numerous juvenile Leydig cells, some of which till remained immature. However, no phases transitional between the two vere observed. Also in the interstitium were groups of Leydig cells that still emained intact after exhaustion of their cytoplasmic lipids (fig. 1, d). In one estis a single mature Leydig cell was dividing (prophase), and in another nimal several such phases were observed.

Epididymides. Some still contained spermatozoa; others were almost mpty except for lymphocytes.

1 May (5 specimens).

Seminiferous tubules. Spermatozoa had disappeared. The tubules had ncreased slightly to about 200 μ in diameter and now contained dividing permatogonia, primary spermatocytes, and secondary spermatocytes. The ext season's spermatogenetic rhythm had already begun. The tubule lumina vere lined with lipid material and basal lipids also remained. The cholesterol eaction was faint and sometimes absent.

Leydig cells. In some vipers the initial stages of spermatogenesis had begun n the presence of only a very small number of mature Leydig cells. Juvenile eydig cells were numerous, but were still only meagrely lipoidal. One mitosis vas observed (fig. 1, e).

Epididymides were empty except for a few lymphocytes.

o June (5 specimens)

Seminiferous tubules. Spermatogenesis was again proceeding strongly with nany rows of germ-cells (including numerous secondary spermatocytes) almost filling tubules measuring about 200 μ wide. Lipids, faintly cholestero positive, were now concentrated in the centre of each tubule except for a fain suffusion at its periphery. Thus sudanophil material was practically absen in the area of pronounced spermatogenetic activity.

Leydig cells. More and more were manufacturing lipids in the form of larg strongly cholesterol-positive globules, but some still remained non-sudanophi Each sort measured about 10 μ in diameter (nucleus 5 μ). Smaller, non lipoidal cells with a nucleus 3 μ in diameter were also common.

Epididymides were empty.

24 July (*9 specimens*)

Seminiferous tubules were as in June, except that some animals alread contained spermatids and had lost all tubule lipids (and therefore cholesterol apart from a few fragments in the narrow central lumina. Some vipers ha whole tubules filled with actively dividing germ-cells (fig. 1, F).

Leydig cells. Here, too, remarkable transitional events were obvious: i some animals the interstitium was still only meagrely lipoidal (yet numerou germ-cells had arisen), while in others (in which the tubule lipids had large vanished) the Leydig cells had become heavily lipoidal and aggregated int tracts as large as 15 by 10 μ (fig. 1, F). These were strongly cholesterol-positiv

Epididymides were empty.

12–18 September (*8 specimens*)

Seminiferous tubules were up to 240 μ in diameter according to the in dividual. Some already contained a few immature spermatozoa. In mo animals tubule lipids had disappeared except for small quantities c cholesterol-negative fragments restricted to the centres. In some they wer absent altogether. In two individuals, however, a minor autumnal steatogenes had begun. The resultant fatty material, which was weakly positive fo cholesterol, showed faintly in the peripheral regions and more strongly in th centres.

Leydig cells were at nearly maximum size (see under March). Many wer losing their lipids and becoming vacuolated. All cytoplasmic lipid materi was powerfully Schultz-positive.

Epididymides were empty.

28 October (*2 specimens*)

Seminiferous tubules were up to 220 μ wide and packed with germ-cell including a few early stages of spermatozoa. Again faint traces of an autumn steatogenesis were discernible.

Leydig cells were heavily lipoidal, and cholesterol-positive aggregation occurred. The cytoplasm of these, however, showed no sign of season depletion.

Epididymides were empty.

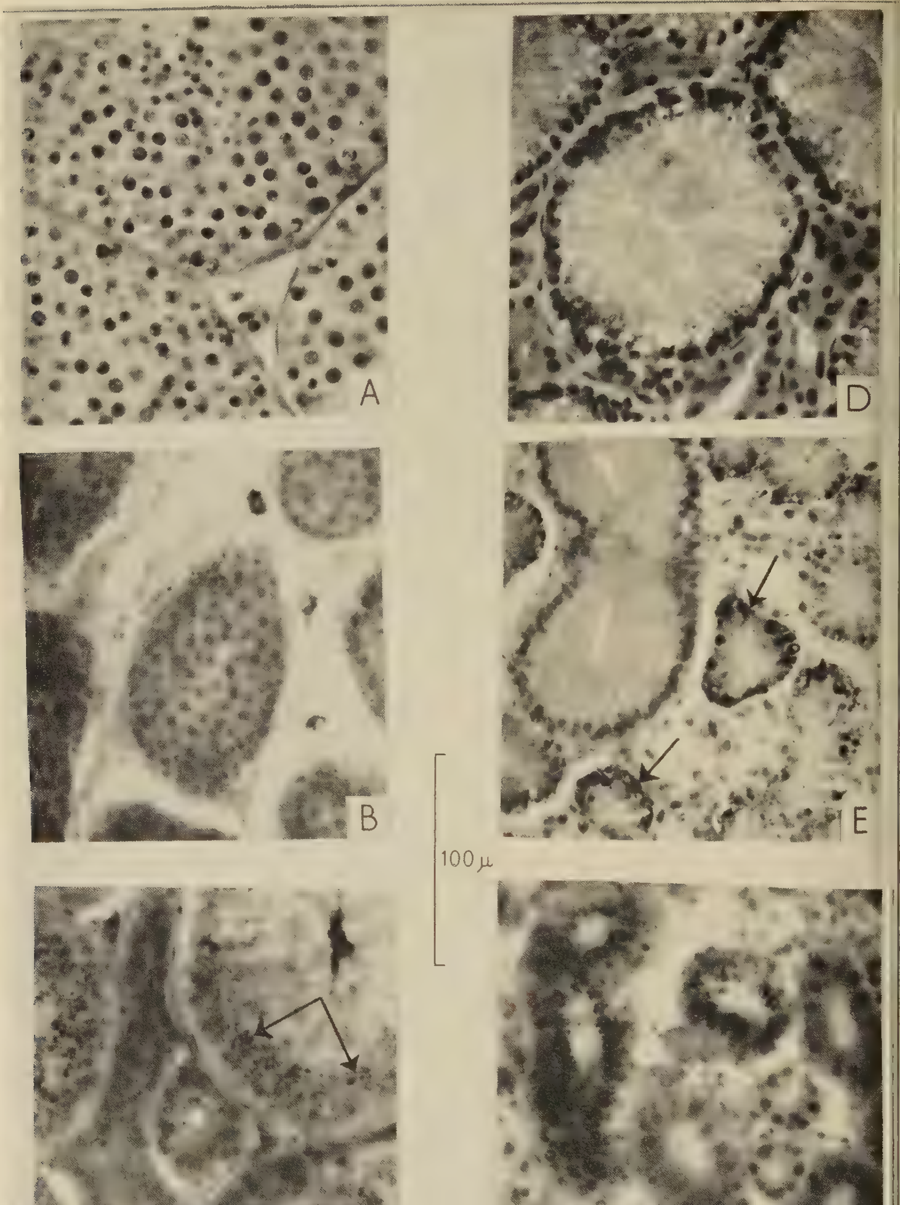

Fig. 2

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November (3 specimens)

Seminiferous tubules were as in October, but with slightly increased steato- nesis, a greater number of spermatids, and a few fully formed spermatozoa.

Leydig cells were as in October except that several mitotic divisions (pro- ase) were seen in one animal.

Epididymides. Despite the presence of tubule spermatozoa the epididy- ides were empty.

January (4 specimens taken from experimental hibernation)

Seminiferous tubules were up to 230 μ in diameter, but it cannot be suggested at the minor apparent increase had occurred during hibernation, because ermatogenesis in each animal was at a halt. The most advanced stage was at of spermatid (fig. 2, A). The tubules were faintly suffused with lipid that as very dubiously positive for cholesterol.

Leydig cells. These were heavily charged with lipid, including cholesterol, d were 12 μ in diameter (nucleus 6 μ).

Epididymides were empty.

veniles taken on 30 June (3 specimens)

Seminiferous tubules were between 50 and 90 μ wide, with spermatogonia division and primary spermatocytes.

Leydig cells were in tracts up to 70 by 50 μ, the individual cells being 10 μ ide (nucleus 5 μ). They were lipoidal and cholesterol-positive (fig. 2, B).

Epididymides were empty.

DISCUSSION

Like those of birds, the testes of the Chelonia, Crocodilia, and Lacertilia ntain Leydig cells of seasonally-variable lipid content and tubules that ndergo a post-spermatogenetic steatogenesis. Therefore, however far modern akes (e.g. *Vipera*) have changed from the ancestral archeosaurian stock om which sprang the Aves, it is probable that such characteristics are erived in both classes from a common ancestry. As might be expected, a

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FIG. 2 (plate). A, 3 January (in experimental hibernation). Testis with all germinal stages up spermatids. Mitotic activity, however, has stopped (compare fig. 1, E). (Technique as in . 1, C.)

B, juvenile (365 mm long) 30 June. Testis with primary spermatocytes and a lipoidal, olesterol-positive interstitium. There has been no advanced spermatogenesis and therefore metamorphosis (compare fig. 1, A, B, D). (Technique as in fig. 1, A.)

C, male kidney (18 May), showing cholesterol-positive lipids in basal epithelium (arrowed) d aggregations of the same material in the tubule lumen. (Technique as in fig. 1, A.)

D, male kidney (18 May), showing depletion of lipid elements. (Technique as in fig. 1, A.)

E, male kidney (18 September), with lipid-free 'sexual-segment', but with apparent renal ements (arrowed) pronouncedly lipoidal and cholesterol-positive. (Technique as in fig. 1, A.)

F, female kidney (20 June), showing an appreciable steatogenesis in the larger elements hich probably correspond with the male 'sexual segment' shown in fig. 2, C, D, E. (Technique in fig. 1, A.)

comparison between the cyclical events in the testis of birds and viper show both similarities and differences.

The points of similarity are:

1. As distinct from the minor lipophanerosis that occurs typically at th end of vertebrate spermatogenesis, the unshed germinal material of bot reptiles and birds metamorphoses into a mass of cholesterol-positive lipid This material gradually disappears, but not down the vas. The cholester may be of endocrine significance; see, however, below, in regard to *Vipera*

2. With the heightening of spermatogenesis and sexual behaviour, th cholesterol-positive lipids of the interstitial Leydig cells are graduall depleted, presumably contributing to the manufacture of the male se hormone. In both reptiles and birds a new, lipoidal interstitial generatio arises after the seasonal exhaustion of the old.

3. Autumnal sexuality occurs in some vipers as also in many avian specie as a consequence of the post-nuptial development and maturation of th interstitium.

Related to the above described common pattern are the following difference in detail:

1. The tubule lipids of the viper are only weakly positive for cholestero and soon after their genesis become almost or wholly negatively so, wherea neighbouring Leydig lipids, whenever present, are always powerfully positive In birds both tubule and interstitial elements are equally and strongl cholesterol-positive. It is impossible in the viper, therefore, to suggest wit any assurance that such briefly-appearing, meagre cholesterol is as functionall important as it may be in birds.

2. In the viper spermatogenesis starts and continues while the tubules ar still clotted with post-nuptial lipids, so that for only a brief transitional perio during autumn is the testis almost without tubule-lipids. In birds such lipid remain for a lengthy period, varying between species, but always disappea abruptly at the onset of the next spermatogenesis, so no trace of them remain by the time large numbers of primary spermatocytes appear. In the vipe (which begins its next spermatogenetic rhythm almost immediately after th preceding one ends), spermatogenesis proceeds strongly beneath a heavy cargo of tubule fat, which has, however, almost disappeared by the tim spermatids have appeared.

3. In the viper the early stages of spermatogenesis (at least up to primary spermatocytes in synizesis) are sometimes achieved while the newly regenerated Leydig cells are still only meagrely lipoidal and at the most only weakly cholesterol-positive. The new interstitium sometimes does not become heavily lipoidal, cholesterol-positive, and mature until several rows of new germ-cells have arisen (in mid-summer). In birds, on the contrary, the new spermatogenesis occurs only after the new interstitium has become relatively mature.

4. In *Vipera* there exist relatively few Leydig cells, but two means (each extremely rare among birds) of increasing their numbers exist. In birds mitotic

division of mature Leydig cells is rare: in *Vipera* it is not uncommon, having been occasionally observed in March (Volsoe), May (fig. 1, E), and November. A second means of increasing the endocrine complement of the testis of the viper is the assumption of a lipid and cholesterol-positive cytoplasm by odd spindle-shaped interstitial fibroblasts during the post-nuptial period of rehabilitation.

5. Almost immediately after the spring shedding of spermatozoa there begins in *Vipera* the succeeding spermatogenetic rhythm—a phenomenon unknown in birds. A broadly comparable rapid recovery of spermatogenetic function occurs, however, in the pike, *Esox lucius* (Lofts and Marshall, 1956) and in the common frog, *Rana temporaria* (van Oordt, 1956). This is almost certainly an adaptation related to the poikilothermic condition and to the thermal fluctuations of the temperate environment. The rapid renewal of spermatogenetic processes ensures that in the viper many secondary spermatocytes, a few spermatids, and often some spermatozoa have been formed before the late autumn hibernation. This is a normal and unvarying component of the sexual cycle, whereas when autumnal spermatogenesis occurs in birds it is wasteful: it generally leads only to a second lipoidal metamorphosis or, occasionally, to an almost invariably unsuccessful winter reproduction. But in *Vipera* the summer spermatogenesis stops during hibernation and, when the snake emerges in the spring, the sexual cycle, already at an advanced stage, begins anew. At the same time fertilization in the viper does not occur earlier than that of most birds, most of which contain merely spermatogonia at the end of January. The high rate of avian metabolism enables birds to achieve in the space of a very few weeks events that in *Vipera* occupy months. The rook (*Corvus frugilegus*), for example, does not begin its annual spermatogenesis until very late in January or early in February, but it copulates by mid-March (Marshall and Coombs, 1956). *Vipera*, on the contrary, emerges when temperatures rise to about 8° C (Volsoe, 1944), basks in the sun as it completes its spermatogenesis, and copulates in April and May.

6. In the viper there is only a minor variation in tubule-diameter and, it follows, total testis size. Similarly, there is apparently no marked and sudden seasonal renewal of the *tunica albuginea* such as occurs in birds and in the pike. At the same time, however, we saw a tunic variation from between 5 and 20 μ in width, and, in some specimens taken from April to July, noted a tendency for the coat to be separated into two layers. The inner and thinner (from 5 to 10 μ thick) appeared to be more densely composed and so it is possible that in this animal too, some tunic rehabilitation occurs at the time when other regenerative events are taking place.

The present investigation, although primarily concerned with the seasonal distribution of testis lipids, has served incidentally to confirm many aspects of the work of Volsoe (1944). We and he are at variance on only two points. Thus, Volsoe writes of the scarcity of interstitial cells in Danish vipers, but we have not found them rare, although it is true that they are fewer than in any bird so far investigated. The supposed rarity and low degree of variation in interstitial cells is seen to be an illusion if we correctly identify the rounded initially non-lipoidal *juvenile* Leydig cells (apparently of connective tissu origin) and, by means of Sudan reagents, follow the seasonal depletion o cholesterol in their cytoplasm after maturity. These, sometimes at leas become pycnotic. The apparent lack of variation is engendered by their capacity for very suddenly reaching their almost, or quite, maximum siz and, as far as we have seen, the persistence of at least some mature cells in th interstitium during hibernation and perhaps (unlike in birds) even after th conclusion of spermatogenesis.

Secondly, we have been unable to confirm with British material Volsoe' statement that 'apart from the pause which occurs during hibernation spermatogenesis is continuous, one spermatogenetic cycle beginning eve before the preceding is concluded'. Although, in a given British population we found individuals still producing the last spermatozoa of the season, an other animals already with the first mitotic spermatogonia of the next rhythm we found beside them still other vipers with reduced tubules containing a fe remaining spermatozoa and aggregations of inactive spermatids—yet with n mitotic divisions whatever (fig. 1, c). The basal layers of such animals wer composed of rows of spermatogonia which showed no sign of modification Therefore, we believe that in some British vipers at least there is a distinct though brief, lull between the end of one spermatogenesis and the start of th next. Very quickly, however, the next cycle begins: the poikilothermous vipe almost immediately takes advantage of the spring and summer warmth. I completes much of its next spermatogenesis before the females have droppe the young of the preceding rhythm.

Although autumn mating in vipers has been observed in Guernsey, an combat in October in Surrey (Smith, 1951), Volsoe found no pregnanc among naturally hibernating females in Denmark. Of the 5 adult female put by us into hibernation on 30 September, one had produced young and second contained embryos of about birth-size when the torpid snakes wer killed on 30 January. These results, however, cannot be said to support th belief of Wolleback (cited and rejected by Volsoe), based on the discover of a pregnant female in Norway late in March, that young may be born durin natural hibernation. From the size of our January embryos and young it i perhaps possible that autumn sexual behaviour might have led to pregnanc just before their enforced hibernation. It seems far more probable, however that our interference retarded the normal autumn parturition and that th females were still gravid from a spring mating when put into darkness. Both Vainio (1931) in Finland and Bernstrom (cited by Volsoe, 1944) in Sweden showed that in higher latitudes there exists an adaptation that allows gestation to continue through the brief northern summer into the next hibernation, s that reproduction occurs every second year.

As with birds of the temperate zone, the sexual processes of *Vipera* ar inhibited by winter conditions, but in the viper the winter cessation o spermatogenesis is not followed by profound retrogressive changes. The few utumn spermatozoa may degenerate, but the relatively advanced germinal tages retain their integrity and the cycle appears to begin without further lelay upon emergence from hibernation. The epididymides of some Scottish ipers were full of massed spermatozoa on 31 March, somewhat earlier than n Denmark. On the other hand, neither Volsoe nor ourselves found evidence f free spermatozoa during the autumn. He found them in the epididymides ntil July. In our snakes they disappeared by the end of May.

Volsoe's information that vipers emerge from hibernation when air temperaures reach about 8° C, and a consideration of their subsequent predilection or basking in the sun make it difficult to resist the conclusion that the sexual ycle is essentially under thermal control. It is obvious, however, that other actors are involved. Spermatozoa in our animals had been shed (i.e. the pididymides were empty) by the end of May and some spermatids of the ext generation were formed by 24 July. In many vertebrates, including pparently the viper in spring, the concluding stages of the spermatogenetic ycle occupy only a matter of a few weeks or, in birds, even days. Although he days remain hot and relatively long between July and September, very ew spermatozoa are formed by the time the vipers, now again probably ontrolled by air temperature, return to hibernation in November. Clearly here has been some intervening resistance to spermatogenesis. As a conequence, and despite widespread autumnal sexual behaviour, fertilization whatever the condition of the female) cannot often take place at the beginning f the winter.

Because of the reported reproductive significance of the 'sexual segment' f the renal tubule, we tested sections of the kidney for lipid and cholesterol. Ve found the epithelium of the wider tubules positive to the Schultz test, but vere unable to correlate the observed cyclical changes with events in the nterstitium. We found, in fact, that reptiles taken at the same period and lace varied considerably in regard to their renal condition, even when the estis elements were in an approximately identical state. It is important to mphasize that much of our material was not gathered by ourselves, and that despite guarantees to the contrary by a professional collector) some of it may ccasionally have remained some time in captivity before dispatch to the aboratory. We found, however, that in the spring the epithelium and lumen f the 'sexual segment' could be heavily spotted with lipids and cholesterol fig. 2, c) when the Leydig cells were either essentially non-lipoidal (fig. 1, a) r heavily so (fig. 1, b). Similarly, there was no clear-cut correlation between he condition of the sexual segment and that of the seminiferous tubules, lthough there was a suggestion in some animals that an expenditure of sexual egment cholesterol occurred during both the spring and late summer periods f spermatogenetic activity.

It was at least certain, however, that a cycle of activity takes place in the enal epithelium itself. Fig. 2, c shows a large 'sexual segment' (arrowed), nottled with lipid droplets (containing cholesterol); and fig. 2, d depicts that f a viper, received and killed on the same day (18 May), vacuolated and depleted of such substances. Fig. 2, E shows another phenomenon not previously commented on: after the clearing of the 'sexual segment', there ha occurred a marked steatogenesis (arrowed) of the smaller and apparentl essentially 'renal segment' of the tubule.

The lipids thus indicated in fig. 2, E were as heavily laden with cholestero as were those of the 'sexual segments' of fig. 2, C. In the female, too, relativel minor changes, involving a similar steatogenesis (fig. 2, F) take place in tha part of the tubule which probably corresponds with the sexual segment of th male. The problem of the cyclical changes in the kidney in relation to externa factors and the gonads invites an experimental analysis with animals from rigidly controlled environment.

We are grateful to Dr. Avrion Mitchison for help in obtaining material One of us (F. M. W.) was in receipt of a grant from the Central Researc Committee of the University of London.

REFERENCES

Coombs, C. J. F., and Marshall, A. J., 1956. J. Endocrin., **13**, 107.

Gampert, O., 1866. Zeit. wiss. Zool., **16**, 369.

Lofts, B., and Marshall, A. J., 1956. Quart. J. micr. Sci., **98**, 79.

Marshall, A. J., 1949. Quart. J. micr. Sci., **90**, 265.

—— 1951. Proc. Zool. Soc. Lond., **121**, 727.

—— 1955. Mem. Soc. Endocrinol., **4**, 75.

—— and Coombs, C. J. F., 1956. Personal communication.

—— and Lofts, B., 1956. Nature, **177**, 704.

Morley, A., 1943. Ibis, **85**, 132.

Oordt, P. G. W. J. van, 1956. *Regulation of the spermatogenetic cycle in the Common Fro (Rana temporaria).* Arnhem (Vander Weil).

Regaud, C., and Policard, A., 1903. Arch. Anat. micr., **6**, 191.

Serventy, D. L., and Marshall, A. J., 1956. Emu, in press.

Smith, M., 1951. *The British amphibians and reptiles.* London (Collins).

Vainio, I., 1931. Ann. Soc. Zool. Bot. Fenn., **12**, 1.

Volsoe, H., 1944. *Structure and seasonal variation of the male reproductive organs of Viper berus (L.).* Copenhagen (Bianco Lunos Bogtrykkeri A/S).

The Growth of the Trout Retina

By A. H. LYALL

(*From the Department of Zoology, Liverpool University*)

With two plates (Figs. 3 and 6)

SUMMARY

1. The growth of the trout eye is negatively allometric in relation to body-length.
2. The relative thicknesses of the retinal layers, and their cell densities, change as the eye grows.
3. The addition of new cells takes place from a peripheral growth zone.
4. Measurements from a wide size-range of trout retinae show that the dimensions of the cone pattern increase as the retina grows.
5. The relative importance of these two processes changes as the eye grows; in early growth the addition of new cells is the principal factor, but the enlargement of the cone pattern, which occurs at an almost constant rate throughout growth, becomes more significant later.

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INTRODUCTION

THE retina of a fish goes on growing throughout its life. Conflicting accounts have been given of how the manifold increase in area is achieved. It is obvious that an increase in area implies either the spreading out of pre-existing elements or the addition of new elements; or both processes may occur together or consecutively. The arrangement of the cones in regular patterns in the retina of a trout offers a convenient means of estimating the relative importance of the spreading out process at every stage of growth; and trout can readily be obtained in an age- and size-range which corresponds to a more than hundredfold change in the area of the retina. It is therefore a particularly suitable species for the investigation here described.

METHODS

Adult trout (*Salmo trutta*) were obtained from Lake Bala and the surrounding streams, and young ones were reared in the laboratory and were also obtained from the Midland Hatchery. The body-length from snout to the fork of the tail was measured on a millimetre rule. The eyes were excised and their antero-posterior diameter measured with a micrometer eye-piece.

[Quarterly Journal of Microscopical Science, Vol. 98, part 1, pp. 101–110, March 1957.]

The measurements of the left eye were always recorded to eliminate any differences between the right and left eyes, although a number of comparisons of the dimensions of the two eyes did not show any differences between them. The measurements of cone-pattern size were obtained from tangential sections of the retinae with a micrometer eye-piece. The distance across ten pattern units was measured and from this the size of one pattern unit was calculated.

In describing the retinal layers I have referred to the surface nearest the chorioid as the external, or outer surface, and that nearest the lens as the inner surface. The region of the retina nearest the iris is described as periphera and that near the point of entry of the optic nerve as central.

Most of the eyes were fixed in Bouin. The outer coats of the eyeball were removed immediately before embedding (except for very small eyes which were embedded whole), and only the retina was embedded in paraffin wax. It is important to keep the iris intact if one wishes to examine the peripheral growth zone of the retina as they are closely associated with each other and the growth zone is easily damaged when removing the retina from the eye. Serial sections 4–8 μ thick were cut in the tangential plane near the pole of the eye to study the cone pattern, and longitudinal sections were cut to examine the periphery of the retina and the distribution of the retinal cell-layers. The sections were stained with Heidenhain's haematoxylin and eosin, Ehrlich's haematoxylin and eosin, and Mallory.

RESULTS

The trout eye shows a considerable increase in size during growth, and this investigation covers a tenfold increase in eye diameter. I have used the body-length as a measure of the size of the fish for comparison with eye-diameter, the larger fish being considered older than smaller ones. If eye-diameter and body-length are plotted on a logarithmic scale, there is seen to be a direct relationship between them over most of the size range (fig. 1). The growth of the eye is negatively allometric in relation to body-length: this is seen most clearly if the relative eye-diameter (i.e. the ratio of eye-diameter to body-length expressed as a percentage) is plotted against body-length (fig. 2), and this shows that large trout have relatively smaller eyes than small ones.

The development of the retina

The rate of increase of eye-diameter, and also of retinal surface area, is greatest in young trout, 15–50 mm long, and at this stage the enlargement of the retina is accompanied by developmental changes involving the relative proportions of retinal layers and cell densities. The vertebrate retina develops from the centre towards the periphery; the innermost layer differentiates first and the visual cell-layer last. Walls (1942) states that the rods and cones differentiate when proliferation ceases. This generalization is not applicable to the whole retina, but, of any particular small region, it is true that differentiation of the visual cells only takes place when cell-divisions in that region have ceased.

The eyes of trout are well developed at the time of hatching and all the retinal layers can be distinguished in longitudinal sections. Fig. 3, A–D, shows four stages in the development of the retina. In the retina of a newly hatched trout (A) the visual cell-layer is represented by a thin protoplasmic-layer between the outer nuclear-layer and the pigment epithelium, and the individual rods and cones cannot be distinguished. The inner nuclear-layer is very thick and can be subdivided into two regions, the outer consisting of darkly stained, elongated nuclei closely packed together, and the inner region of paler, round nuclei. The inner fibre-layer is relatively thin at this stage and the ganglion cell-layer has three or four layers of nuclei.

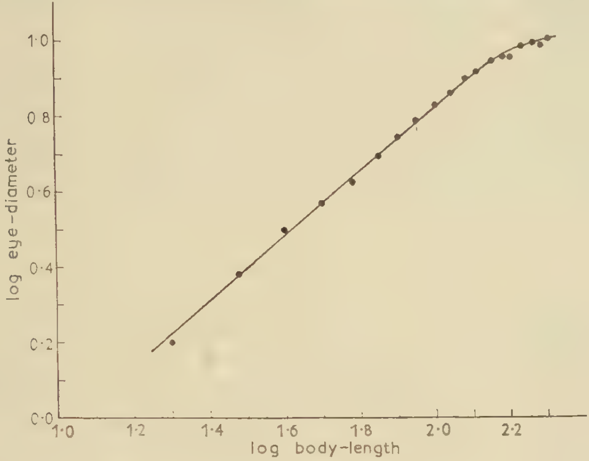

FIG. 1. Eye-diameter / body-length plotted on a logarithmic scale.

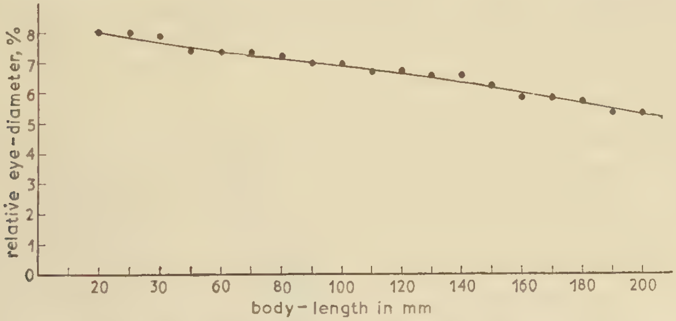

FIG. 2. The relative eye-diameter (eye-diameter / body-length percentage) plotted against body-length.

The most marked change during the early development of the trout retina is the great increase in thickness of the visual cell-layer. The rods and cones develop during the first week after hatching but their outer segments do not quite reach to the pigment-cells at this stage. The visual cells grow rapidly during the first month after hatching and then increase in size more slowly. The outer nuclear-layer increases in thickness during later growth. There are only two layers of rod nuclei in young retinae but in an adult retina there are three rows. The origin of these extra rod nuclei in the outer nuclear-layer is uncertain, but it seems probable that undifferentiated cells are present in the inner nuclear-layer of young retinae and that these migrate into the outer nuclear-layer where they differentiate to form rods. The inner nuclear-layer decreases in absolute thickness until about 6 weeks after hatching, after which it remains approximately the same but the nuclei become more spread out. The distinction between the two types of nuclei in this layer becomes less marked, but the outer nuclei are still distinguishable by their darker staining properties 3 months after hatching. The inner and outer fibre-layers increase in thickness as the retina grows and this is particularly noticeable with the inner fibre-layer during the first 2 months. The ganglion cell nuclei become more spread out as the retina grows; 3–4 weeks after hatching there are only two layers of these nuclei in the central regions of the retina and after another 2 or 3 months they form a single layer and later become more widely dispersed. The increased thickness of the retina in an adult trout, compared with that of an alevin, is largely due to the greater development of the visual cell- and fibre-layers.

Mitoses

During very early development mitoses occur over the whole trout retina, but when differentiation commences near the centre of the retina, divisions in that region cease; and finally the whole retina differentiates except at the extreme edges where mitoses persist and constitute a peripheral growth zone. In young trout retinae mitoses still occur along the edges of the falciform process when they have disappeared from the surrounding areas. I have examined longitudinal sections of trout retinae of various sizes (1·2 mm to 11·6 mm eye-diameter) and I have found mitoses in the periphery throughout the size-range. The mitoses are usually situated immediately beneath the pigment epithelium in the undifferentiated peripheral region of the retina (fig. 3, E). In two or three retinae I have found a mitosis some distance from the edge of the retina in the outer nuclear-layer, in a region where differentiation is completed and the rods and cones have well-developed outer segments. These mitoses were found in the eyes of fish 1–2 years old, so a few nuclei in the outer nuclear-layer must retain the power to undergo mitosis.

I have generally cut each retina in only one longitudinal plane, but by cutting different retinae in different planes I have found cell-divisions in all radii, and mitoses must occur around the whole circumference of the growth

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FIG. 3 (plate). A D, stages in the development of the trout retina: portions of longitudinal sections of retinae from fish of different ages. A, newly hatched; B, 6 weeks old; C, 3 months old; D, adult (depigmented).

E, part of a longitudinal section of a young trout retina showing mitoses in the peripheral growth zone.

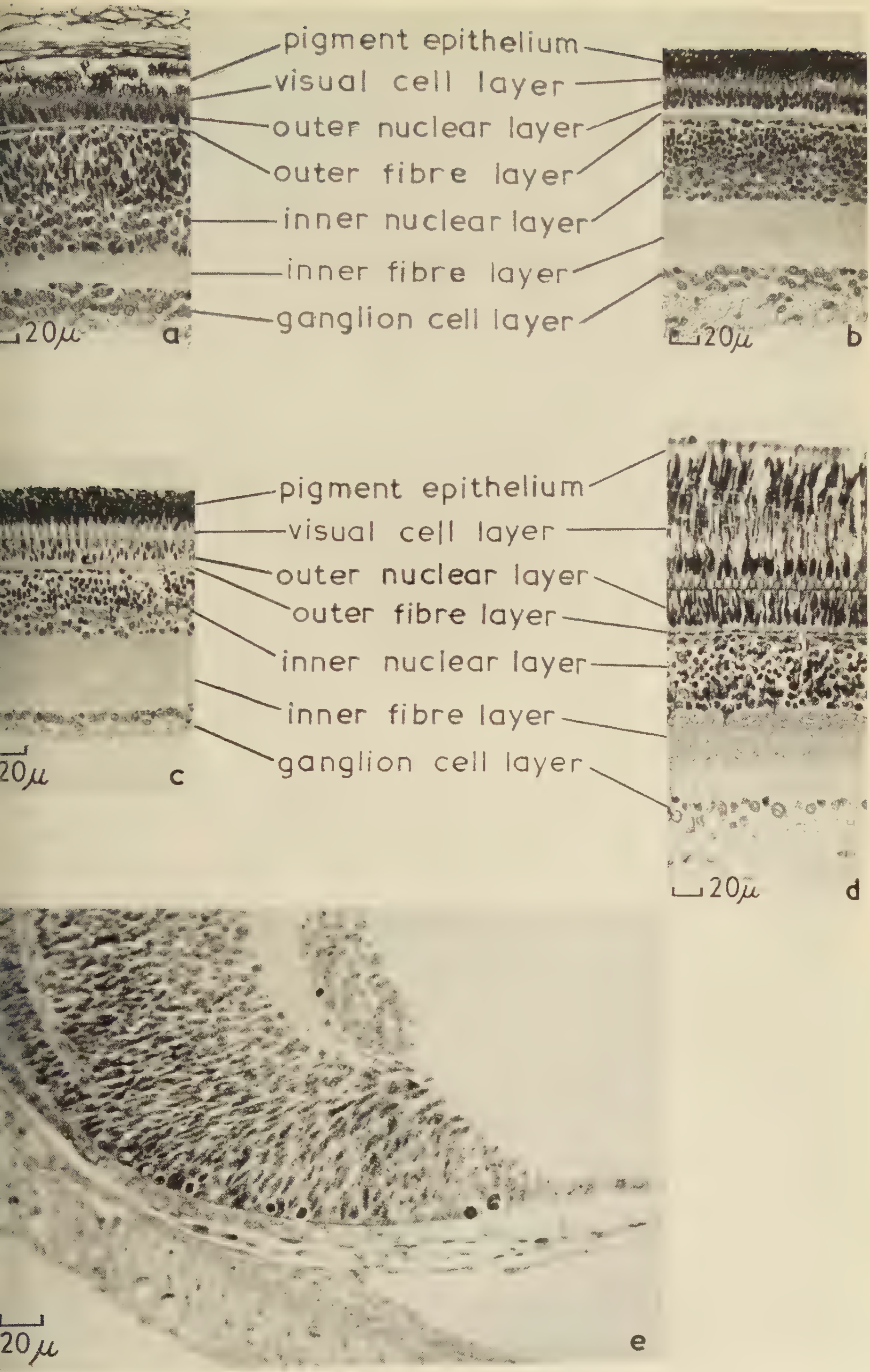


FIG. 3

A. H. LYALL

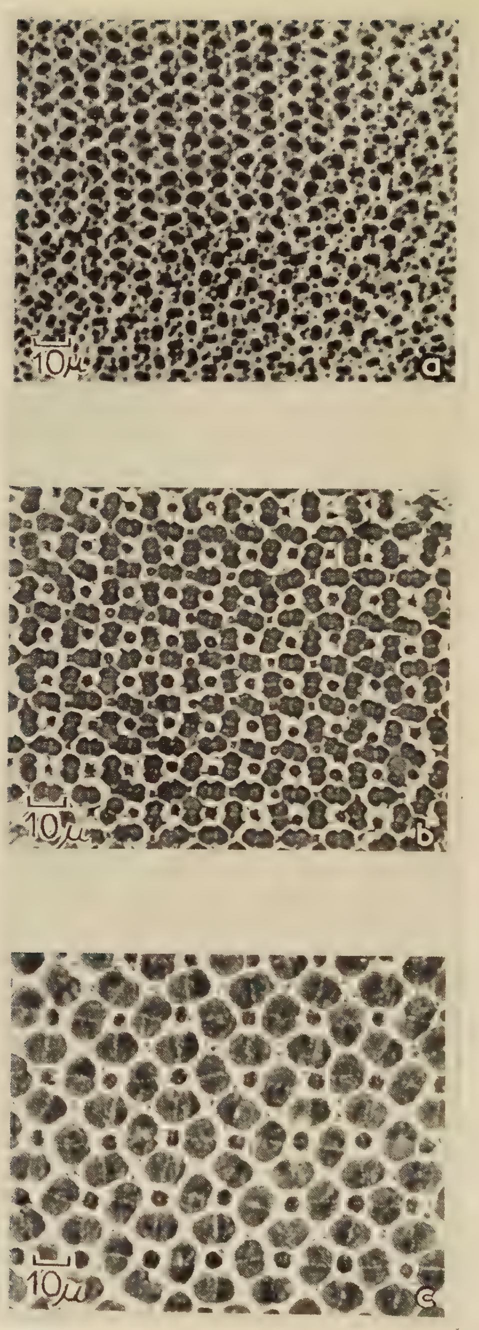


Fig. 6

A. H. LYALL

one. The mitoses are not uniformly distributed: in a series of longitudinal sec-ons the divisions may be numerous in a number of sections and then entirely bsent from the succeeding 12 or more sections. This irregular grouping of he dividing cells makes comparisons of the number of mitoses in different etinae unreliable, and also comparisons between different regions of the same etina. If, however, two eyes of very different size are compared, there is no oubt that mitoses are more numerous in the smaller eye. The persistence of itoses in adult trout retinae indicates that new cells are still being formed nd, although there are relatively few divisions, the increase in the number of ells will contribute to the growth of the retina.

he visual cell patterns

There is a regular arrangement of cones in trout retinae of all ages, and the one pattern is of particular interest when considering the increase in retinal urface area during growth. In very young trout there is a regular arrange-ent of rods and cones which can be seen in tangential sections of the retina. he cone pattern consists of quadrilateral units formed by four double cones urrounding a central single cone, with an additional single cone at each orner (fig. 4). These units are arranged in rows forming parallel lines of ones intersecting at right angles. In very young trout retinae there are usually our rods arranged symmetrically around the central single cone of each attern unit (fig. 5), but it is not always easy to distinguish the central single one from the rods at this stage in tangential sections, as they have similar iameters in their central regions (fig. 6, A). This rod pattern is only present very young trout retinae: the number of rods per pattern unit soon increases

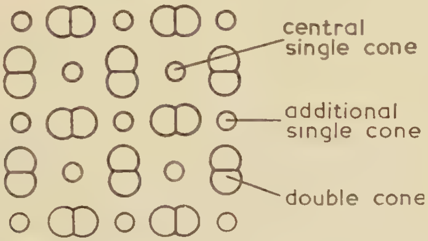


FIG. 4. Diagram of the cone pattern in a young trout retina.

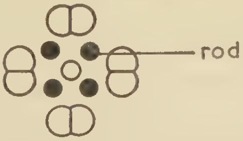


FIG. 5. Diagram showing the rod pattern found in very young retinae.

FIG. 6 (plate). A, tangential section of a very young trout retina showing the rod and cone tterns.

B, tangential section of a young trout retina showing the pattern drawn in fig. 4.

C, tangential section of the retina of a large adult trout showing the cone pattern.

and there is an irregular grouping of rods around the central single cone. T average number of rods per pattern unit in trout with eye-diameters of 4 m and 8 mm is 7 and 12 respectively. The rod arrangement in adult retinae seen most clearly in tangential sections of dark-adapted retinae because in light-adapted eye the myoids of the rods are usually too thin to be identifi in sections through the cone inner segments.

The cone pattern in an adult trout retina is slightly different from th shown in fig. 4. The additional single cones disappear, so that the typic adult trout cone pattern is that shown in fig. 7. The additional single con do not disappear simultaneously and, like the differentiation of the retina, th pattern change-over begins near the pole of the eye and progresses towar the edge. The loss of these single cones does not alter the arrangement the other cones, which remains constant throughout subsequent retin growth. The distance between two central single cones (*a–b*, fig. 7) has bee used as a measure of the cone-pattern size, and this is not affected by th presence or absence of the additional single cones.

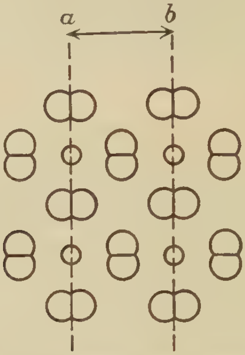
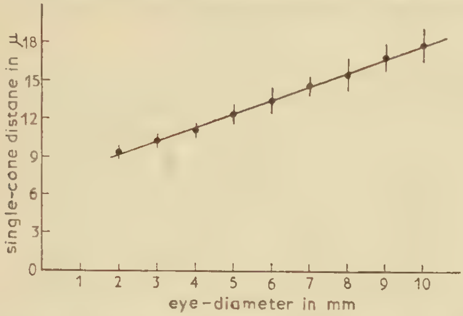


FIG. 7. Diagram of the adult trout cone pattern. The distance *a–b* represents the single-co distance.

The increase in cone-pattern size

The cone-pattern size has been measured in many trout retinae from wide size-range, and it is evident from the results that the dimensions of th pattern increase as the eye grows. Fig. 6, B and C, shows tangential section from a young and a large adult trout retina respectively, and, in addition to th marked difference in the single-cone distance, these sections show that th individual cone elements also increase in size. The relationship between th cone-pattern size (as represented by the single-cone distance) and eye diameter is shown in fig. 8. The pattern enlarges as the eye-diameter increase and the rate of change of pattern size during the growth of the eye is nearl constant. Any deductions from the relationship between pattern size an eye-diameter, concerning the growth of the retina, are made on the assumptions that the single-cone distance is proportional to the square root of th rface area of one pattern unit, and that the eye-diameter is proportional to e square root of the total retinal surface area.

These assumptions involve certain inaccuracies in the calculations of retinal eas. In the first place, the area of one pattern unit is not a flat surface and erefore its surface area is not equal to the square of its single-cone distance, t the curvature of one unit is so small that the errors will be negligible when e area of one pattern unit is considered proportional to the single-cone stance. Much greater inaccuracies are associated with the assumption that e total retinal area is proportional to the square of the eye-diameter. For is to be true, the retina would have to retain the same shape throughout owth. In fact the retina is nearly hemispherical in large eyes, but in young out the eyes are slightly elongated in the antero-posterior direction. The se of the eye-diameter instead of the retina-diameter probably causes the eatest inaccuracies, but a number of measurements of retinal diameters ve been made from longitudinal sections, and the retina-diameter and eye-ameter are directly proportional to each other. These values of retina-ameters suggest that the use of eye-diameters does not affect the general lationships between the change in cone-pattern size and the growth of the tina, although it may alter the apparent rates of change of pattern size.



G. 8. The relationship between the single-cone distance and eye-diameter. (The vertical lines are proportional to the standard deviations.)

Fig. 8 shows that for a five-fold increase in eye-diameter the single-cone stance is only doubled; it follows that at the end of this period less than 0% of the total assembly of double cones and central single cones were esent at the beginning and more than 80% have been added during growth. g. 9 shows that the relative single-cone distance (i.e. single-cone distance / e-diameter %) is not constant in trout retinae and that it decreases as the e-diameter increases. The increase in cone-pattern size is therefore not fficient to account for the increased retinal area and new cells must be added, rticularly in the young eyes where there is a marked decline in the relative ngle-cone distance (fig. 9). In the larger eyes, where the slope of the graph g. 9) is nearer the horizontal (and therefore the number of pattern units is proaching constancy), the increase in retinal area must be largely due to e increase in cone-pattern size.

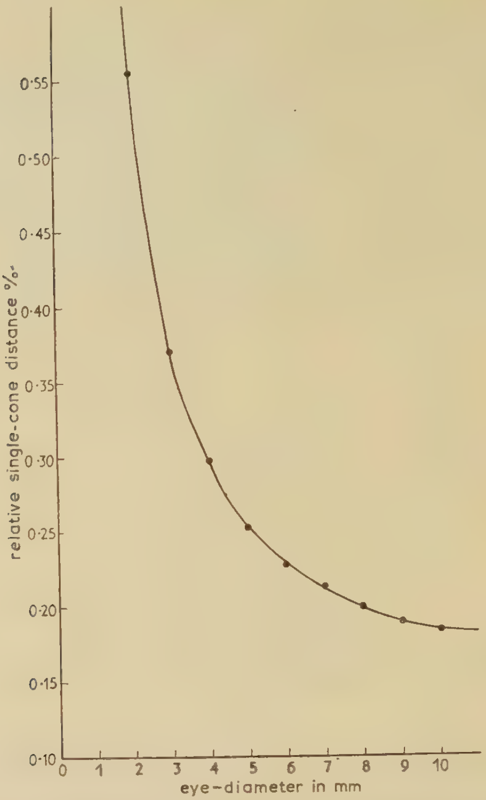


FIG. 9. The relative single-cone distance plotted against eye-diameter.

DISCUSSION

An increase in surface area is the most noticeable feature of post-embryon growth in trout retinae. During the first few months of development, th increase in surface area is accompanied by conspicuous changes in the relativ proportions of the different retinal layers, but these changes do not affect th regular arrangement of visual cells which has been studied in relation to th increased retinal area. Post-embryonic growth in teleosts has been studied i *Micropterus* by Shafer (1900), and in more detail by Müller (1952) in *Lebiste* Shafer studied the cone-pattern size in two different-sized specimens o *Micropterus* and, from a comparison of the ratio of the squares of the patter size with the ratio of the squares of the eye-diameters, he concluded that th enlargement and spreading out of the cones was sufficient to account for th increased retinal area and that no new elements were added. In view of th work reported here, based on a large number of measurements on a wide an continuous size-range, it is probable that Shafer was mistaken. It is clear tha in trout at least, both addition and spreading contribute to the increase i retinal area, but that the latter contributes proportionately more in olde sh. It would therefore have been possible to reach a conclusion similar to hafer's if measurements had been made on two trout only, neither of which as very young.

Müller determined that the growth of the eye of *Lebistes* was negatively lometric and that the retina grew by the addition of cells from a peripheral rowth zone. *Lebistes* has a cone pattern similar to that found in young trout tinae and, although he did not measure the size of the pattern in different sh, he observed that the cones were more spread out in the larger eyes. The rowth of the retina in *Lebistes* is thus due to the addition of new cells and to e spreading out of existing cells, which agrees with my observations in trout tinae, and a combination of these two factors is probably typical of retinal rowth in teleosts.

I have found that dividing cells are present at the edge of trout retinae roughout growth, but the presence of mitoses and a definite growth zone ave not always been recognized, even in the early developmental stages of leost retinae. Wunder (1925) could find no region of increased nuclear ivision during the development of trout and carp retinae, but he believed hat multiplication of the cells must nevertheless occur. Mitoses in trout tinae are confined to the peripheral growth zone, except in very young eyes, nd new cells must be added at the edge. The peripheral position of the rowth zone does not necessarily signify that all new cells differentiate at the dge, as some cells may migrate into more central regions before differen- ating. The uniformity of the cone pattern in trout retinae shows that an crease in the number of cones cannot take place by the addition of new cells roughout the retina, as this would upset the regular arrangement; therefore ew cones must differentiate to form new pattern units at the edge of the tina. The result of this centripetal growth of the trout retina is that each art becomes more central as the eye enlarges. Kolmer (1936) claims that tinal growth in teleosts is appositional, and although this is probably true f most retinal cells in trout, the addition of new rods is not confined to the dge and takes place throughout the retina. The number of rods per pattern nit increases as the retina grows and, since the mitoses are almost entirely onfined to the edge of the retina, the additional rods cannot be formed by ivision of cells in the differentiated regions of the retina. The migration of ndifferentiated cells into the outer nuclear layer is probably responsible for e increase in the number of rods.

The measurements of the cone-pattern size in trout retinae show conlusively that the pattern increases in size as the eye grows and this will ontribute to the increased area of the retina. It has been shown that the rate f increase of the cone-pattern size is almost constant throughout growth, but uring early growth this accounts for only a small fraction of the increased rea and the addition of new cells from the peripheral growth zone contributes ore to the growth of the retina. This has been deduced from figs. 8 and 9 nd is also confirmed by the greater number of mitoses found in young tinae. During later growth, when the mitotic rate has decreased, the spreading out of the cones is more significant. The spreading out of the visu cells decreases the cone density but this is compensated by growth of the le and other parts of the eye so that the size of the image on the retina is all increased.

I am very grateful to Professor R. J. Pumphrey, F.R.S., for his invaluab assistance with this work, which was carried out while I held a D.S.I. maintenance grant. I should also like to thank Dr. J. W. Jones for providin the trout from Bala and Mr. W. Irvine for taking the photographs.

REFERENCES

KOLMER, W., 1936. In Mollendorff's *Handbuch der mikroskopischen Anatomie des Mensch* vol. 3, part 2. Berlin (Springer).
MÜLLER, H., 1952. Zool. Jb., **63**, 275.
SHAFER, G. D., 1900. Arch. EntwMech. Org., **10**, 685.
WALLS, G. L., 1942. *The vertebrate eye.* Michigan (Cranbrook Press).
WUNDER, W., 1925. Z. vergl. Physiol., **3**, 1.

The Male Genital System of the Scorpion, *Buthus quinquestriatus*

By A. ABD-EL-WAHAB, PH.D.

(*From the Institute of Animal Genetics, West Mains Road, Edinburgh*)

With one plate (fig. 5)

SUMMARY

1. The paper consists of a morphological and histological description of the male enital system of the scorpion, *Buthus quinquestriatus* (H.E.).

2. There is a well-developed ganglionic mass at the extreme end of the lumen of the ylindrical gland. This ganglionic mass has not been recorded by any of the previous uthors.

3. There are two pairs of annex glands opening into the common genital chamber. one of the previous authors have observed such a gland with the exception of avlovsky (1915), who recorded it in *B. australis*.

4. New methods have been adopted to resolve the structure of the ejaculatory organ nd its supporting shaft. The organs were cut in serial transverse sections, and whole ounts were also made.

5. In the supporting shaft, several basal processes have been found, namely, the blique vertical, longitudinal, superior, and inferior outer and inner processes.

6. The supporting shaft is found to be of a chitinous nature.

INTRODUCTION

THE male genital system of scorpions, in general, has been described by several authors, e.g. Dufour (1856) and Pavlovsky (1915, 1917, 1921). owever, the accounts available in the writings of these authors are neither recise nor complete. In this paper I wish to contribute a detailed study of e male genital system of *Buthus quinquestriatus* (H.E.), which is the commonest Egyptian scorpion. It has not been investigated by any of the previous uthors.

The material used for this study was collected near Cairo and brought to dinburgh for investigation in the laboratories of the Institute of Animal enetics, University of Edinburgh.

Some specimens were preserved in 70% alcohol for dissection while others ere fixed immediately after being captured in hot Bouin's, Carl's, Carnoy's, nd Sanfelice's fluids. Of these fixatives, Sanfelice's gave the most satisfactory esults for demonstrating the different stages of spermatogenesis in the wall f the seminal tubes.

In dehydration, absolute alcohol and xylene were avoided, since they ender the chitinous organs very hard and impossible to cut into serial sections. learing was done in methyl benzoate and benzene, and embedding in hard ax containing ceresin and stearic acid. The dyes used were Heidenhain's nd Ehrlich's haematoxylins with eosin as a counterstain. Chlorazol black was so used for staining the chitinous organs, as recommended by Cannon 937, 1941).

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GENERAL STRUCTURE

The male genital system of *B. quinquestriatus* (fig. 1, A) consists main of two testes, from each of which emerges a vas deferens. This leads to a seri of accessory organs attached to an ejaculatory organ which unites anterio with the corresponding one to form a very short common chamber openi ventrally to the exterior. The male genital opening is situated posterio between the two genital opercula, which are considered by previous autho (see Abd-el-Wahab, 1952) as the appendages of the first mesosomatic segmen

THE TESTES

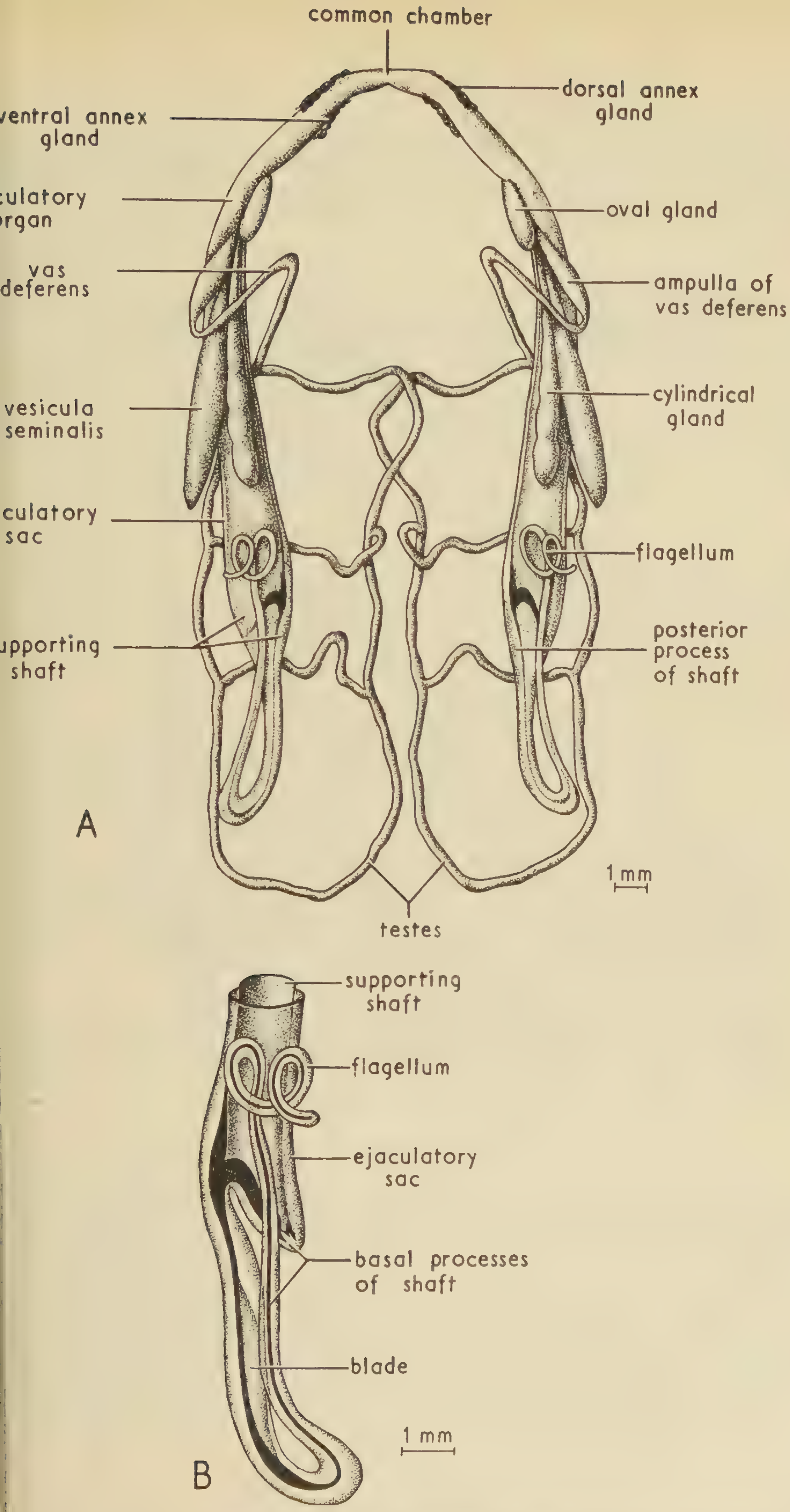
Each testis (fig. 1, A) is composed of several slender tubules which inte communicate with each other to form a ladder-like structure, which is lodge between the inferior lobules of the hepatopancreatic gland ('liver'), in th region of the last five mesosomatic segments. In each testis there are tw longitudinal tubules and four transverse ones, anastomosing with each oth in such a manner that three quadrilateral meshes are formed. One of th longitudinal tubules of each testis runs near the middle of the body and o along the inner border of the mesosomatic cavity. Both the longitudinal a the transverse tubules are extremely delicate, flexible, transparent, white, a of comparatively narrow and uniform calibre throughout their whole lengt In section, these tubules are circular or oval in shape, measuring from abo 0·2 to 0·5 mm in diameter.

The literature shows that the testes differ in structure from one species the other and even in individuals belonging to the same species. The abov mentioned account of the testes of the species under consideration is near similar to what was reported by Dufour (1856) on *Scorpio occitanus*. It differ however, from the reports given by Duvernoy (quoted by Dufour, 1856) c *S. occitanus*, Pavlovsky (1915, 1921) on *Buthus australis* and *Scorpio mauru* and Sato (1940) on *Buthus martensii*.

Duvernoy mentioned that the two testes of *Scorpio occitanus* are united i the middle, as is the case in the ovary of the female. It is probable th Duvernoy's was an abnormal specimen, since Dufour did not find th union in more than 100 individuals of this species.

Pavlovsky (1915) found in some forms of *Buthus australis* two well-develope blind diverticula, one at each end of the median longitudinal tubule of eac testis. He added that there may be two quadrilateral meshes instead of thre in each testis. The same author (1921) reported that in *Scorpio maurus* th testis is not in the form of a ladder but is made only of a single longitudin tubule provided with four blind diverticula projecting from its inner sid He added that the first diverticulum or even the first two diverticula of eac testis may partially unite with the corresponding ones of the other side.

Sato (1940) gave a very brief account of *Buthus martensii*. He showed b figures that the testes are united by a short transverse tubule connecting th first pair of meshes.



. A, the male genital system of *Buthus quinquestriatus*. B, posterior portion of the ejaculatory organ.

Histologically, the wall of the testis (figs. 2; 5, 1) is made of a flatten germinal epithelium resting on a very thin basement membrane, which followed by an extremely thin circular muscle-layer. Inside the membra propria, the primordial germ-cells of the germinal epithelium have given ri

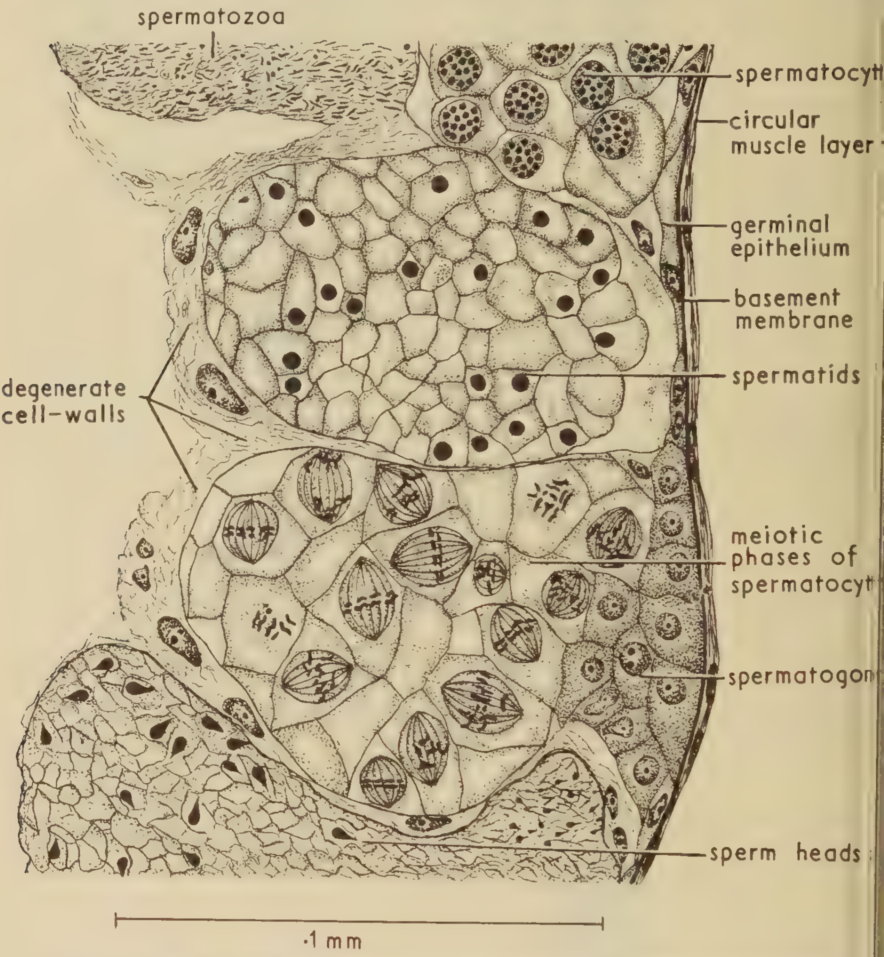


FIG. 2. A portion of a longitudinal section of the testis of *Buthus quinquestriatus*.

to a large number of spermatogonia which are in the form of loose masse small, rounded cells containing a small amount of cytoplasm and dens chromatic nuclei. Some of these masses have been replaced by spermatocy or spermatids or spermatozoa. Each group of similar cells is enclosed b thin wall in the form of a cyst. All the cysts lie closely adjacent to each otl Sometimes the cysts are partially surrounded by several degenerating c walls with oval nuclei.

The spermatocytes are usually larger than the spermatogonia and cont rge nuclei. The spermatids form compact bundles inside their cysts. The usters of sperm heads are surrounded by diffuse and apparently degenerate asses of cytoplasm. The spermatozoa, after becoming mature, are set free to the lumen of the testis, from which they go in clusters through the vas eferens to the seminal reservoirs.

This description of the histology of the testis is nearly similar to that of ato (1940) in *B. martensii*, but differs in some respects from that of Sokolow 913) in *B. eupeus* and *Euscorpius carpathicus*. According to Sokolow's escription, the testis is limited externally by a very high, columnar epithelium. he inner end of every cell of this epithelium stains more strongly than the st and contains a clear vesicle. All these vesicles together form a clear border the epithelium. The spermatogenetic cysts are scattered between the cells the columnar epithelium.

THE VASA DEFERENTIA

There are two vasa deferentia, coming out from the outer angles of the remost mesh of the testis (fig. 1, A). Each vas deferens runs forwards to join ith the vesicula seminalis and the other accessory organs that will be menoned later. It is composed of two main portions, a proximal and a distal. he proximal part is longer than the distal. It is also folded and slender, being early of the same calibre as the testes. The distal part is somewhat dilated. is generally referred to as the terminal 'ampulla'. It is absent in some orpions such as *Buthus australis* and *Scorpio maurus* (see Pavlovsky, 1917, 21).

The wall of the vas deferens in the proximal region is formed of cuboidal lls resting on a basement membrane; there is a thin layer of circular muscle. the wall of the terminal ampulla the epithelium is thrown into folds comsed of cylindrical cells of different heights. The muscle-layer is more onounced and made of intermixed circular and longitudinal muscle fibres.

THE ACCESSORY ORGANS

The terminal ampulla of each vas deferens opens into a series of accessory gans which are generally referred to as the vesicula seminalis and the lindrical and oval glands. The latter open directly into the ejaculatory organ.

ie vesicula seminalis

This is a yellowish organ, nearly club-shaped in form. It is connected by tip to the terminal portion of the dilated ampulla of the vas deferens and s freely over the cylindrical gland and ejaculatory organ, with a slight viation outwards.

It is usually filled with a large mass of spermatozoa (fig. 5, B). Its wall is mparatively thin, being made of flattened cells except near its attachment the vas deferens, where the cells are cuboidal. The cells of the epithelium t on a thin basement membrane; there is an extremely thin layer of interngled muscle fibres.

The cylindrical gland

This gland is a transparent, white, elongated sac, nearly tubular in form It is slightly shorter than the vesicula seminalis and lies closely over th dorsal surface of the ejaculatory organ, to which it is attached by connectiv tissue fibres. It is connected with the dilated portion of the terminal ampull of the vas deferens at a point just beyond the attachment of the vesicul seminalis. Its posterior half is apparently segmented and its posterior end i bulb-like in form.

The wall of the cylindrical gland (fig. 5, B, cg) consists of a cylindrica epithelium of varying height. From the mid-dorsal surface of the posterio two-thirds of the gland, well-developed folds are produced. The muscle layer differs in thickness in the different regions of the gland. It is thick an composed of intermixed circular and longitudinal muscle-fibres.

There is a well-developed ganglionic mass (fig. 5, B, gn) situated at th extreme end of the lumen of the gland. From this mass arise lateral and media nerves running to the wall of the gland itself and that of the ejaculatory organ This ganglion has never been recorded by any of the previous authors.

The oval gland

This is the most anterior gland. It is transparent, white, and nearly oval i form. It receives its contents from the terminal ampulla of the vas deferen at an opening very near to that of the cylindrical gland. It lies towards the inne side of the ejaculatory organ into which it opens. Histologically, its wall nearly similar to that of the cylindrical gland.

THE EJACULATORY ORGAN

Dufour (1856) referred to this organ in *Scorpio occitanus* as the 'penis' 'ejaculatory canal', in the belief that this organ is used in the process of copu lation. However, Birula (1910) claimed that it is not used in copulation. He di covered in *S. maurus* two external papillae acting as the penis during th process of copulation. Accordingly, Pavlovsky (1917) replaced Dufour names by the term 'paraxial organ', owing to the lateral position of the orga

In the present description it is found preferable to use the name 'ejaculato organ', from homology with the corresponding structures in related anima The term paraxial organ is vague, since it can be applied to any other later organ in the mesosomatic cavity. Some scorpions, such as *Buthus austral* *B. leptochelys*, *B. occitanus*, and the species under consideration, do n possess Birula's papillae for copulation.

In *B. quinquestriatus* the ejaculatory organ (fig. 1, A, B) consists of ejaculatory sac supported from inside by a chitinous shaft, which Pavlovs (1921) referred to as the 'supporting stalk' ('tige de soutien').

The ejaculatory sac

This is an extremely elongated hollow structure which is nearly white colour. It extends longitudinally along the inner border of the first five mes somatic segments. It is about 15 mm long and from 1 to 2 mm broad. It is covered almost wholly by the outer border of the hepatopancreas. Its base, from the outer side, is produced slightly backwards and outwards into somewhat swollen and blind epithelial pockets, into which are inserted the basal processes of the supporting shaft (figs. 1, B; 4, B), which will be described later.

From the inner side the base of the ejaculatory sac extends backwards as a slender, vermiform structure which is generally referred to as the flagellum (fig. 1, B). This organ is folded. The proximal part of the fold is thicker and shorter than the distal one, which is in the form of a slender blind tubule coiled at its free end. According to Pavlovsky (1921) such a structure is absent from the ejaculatory sac of *Scorpio maurus*. It is present in most of the species belonging to the family Buthidae (e.g. *Buthus australis*, *B. leptochelys*, and *B. occitanus*).

The two ejaculatory sacs unite and form a comparatively short common chamber whose breadth exceeds its length (fig. 1, A). This is situated transversely and in its lumen there is a median fold projecting from the dorsal wall (fig. 5, A, fo). Ventrally the common chamber opens to the exterior by a narrow genital opening situated between the posterior margins of the genital opercula.

Opening into the common chamber from each side is a single pair of minute glands, one dorsal and one ventral, which are generally referred to as the annex glands. These yellowish glands are much branched. From their openings they extend backwards, closely adjacent to the wall of the common chamber. None of the previous authors has noticed such glands except Pavlovsky (1915) in *B. australis*. The function of such glands is still unknown, though it may be suggested that they secrete a substance to lubricate the vagina of the female during the process of copulation.

The wall of the ejaculatory sac (fig. 3, A, B) consists of a simple epithelium resting on a thin basement membrane. There is a thick layer of muscle-fibres. The epithelium is made of cylindrical cells which differ in height in the different regions of the organ. At the places where the margins and processes of the supporting shaft are inserted, the cells are very low. The muscle-layer differs in thickness in the different regions of the ejaculatory sac. It consists of longitudinal muscle-fibres (fig. 3, A). However, it is composed of intermixed circular and longitudinal muscle-fibres in the region of the common-chamber (fig. 3, B).

The wall of the annex gland is of the same structure as that of the ejaculatory sac, with the exception that its epithelium is thrown into more folds and the muscle-layer is rather thin and composed entirely of circular muscle-fibres (fig. 3, B).

The supporting shaft

New methods were adopted to resolve the structure of the supporting shaft. The organ was cut in serial transverse sections. Whole mounts of two

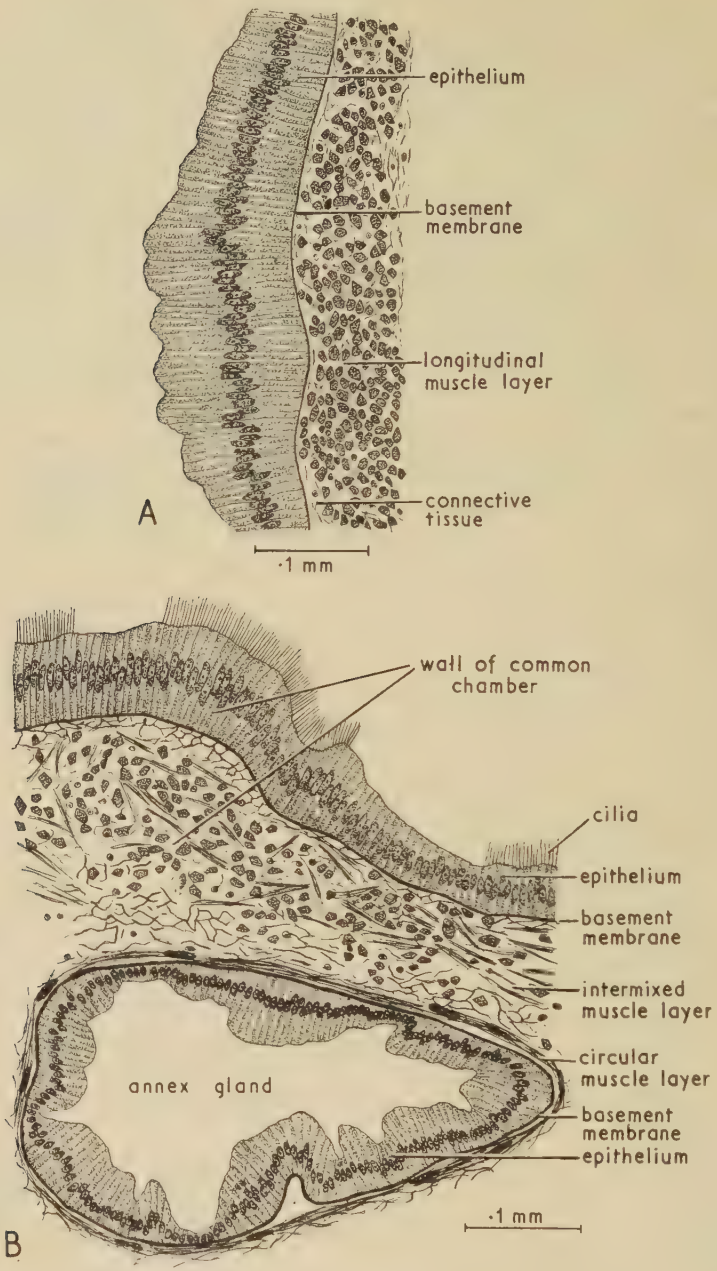


FIG. 3. Two portions of transverse sections through the ejaculatory organ of *Buthus quinquestriatus*. A, in the region of the ejaculatory sac. B, in the region of the common chamber.

kinds were also made. (1) The organ was softened in hot 4% caustic potash and examined in this medium. (2) It was dehydrated, cleared, and examined in Canada balsam.

The shaft consists of a gutter, to which are attached several processes (fig. 4, A). It is a brown structure which is inserted into the lumen of the ejaculatory sac and the common chamber.

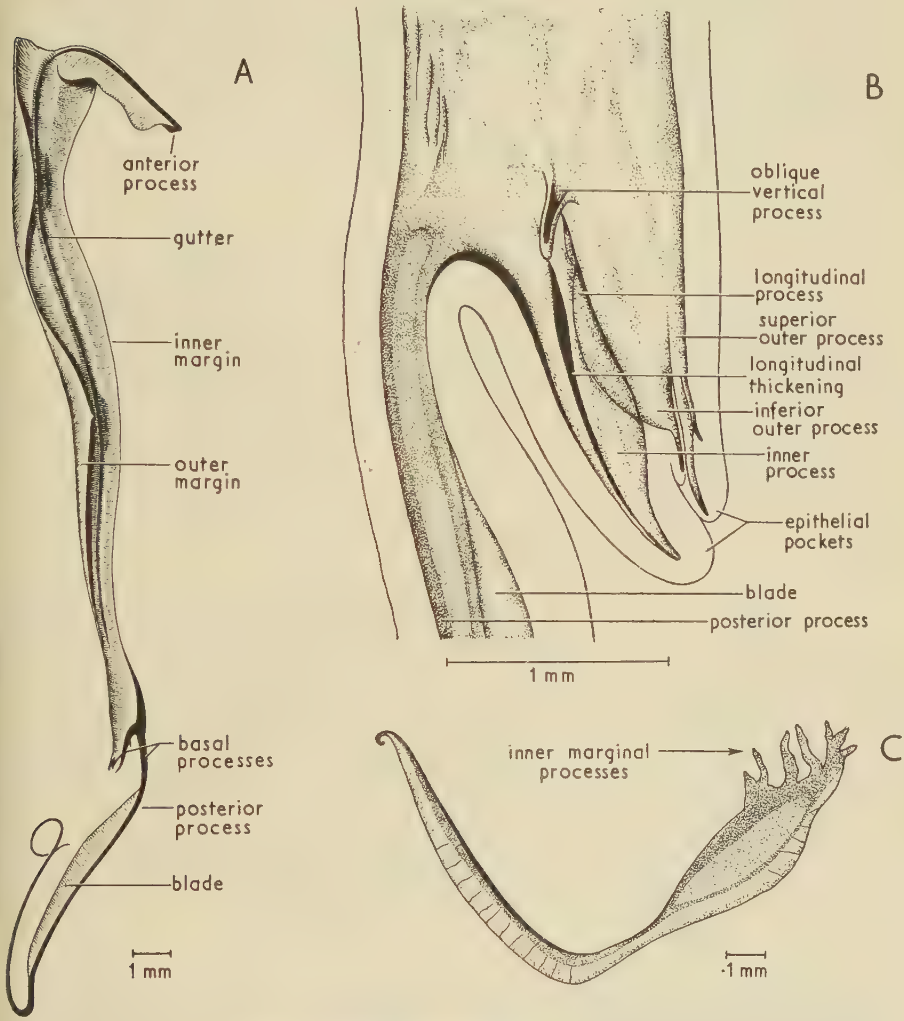


FIG. 4. The supporting shaft of *Buthus quinquestriatus*. A, whole organ after treatment with caustic potash. B, basal processes of the shaft. C, transverse section through the beginning of the last quarter of the shaft.

The *gutter* is a trench-like, chitinous structure whose form differs in the different regions of the ejaculatory sac. In the first three-quarters of the latter, its wall is slightly evaginated but this evagination decreases gradually backwards until it disappears. In other words, the gutter possesses two channels running close to each other in the first three-quarters (fig. 5, B) and the wall between these channels decreases gradually in height until one finds only a single channel in the last quarter (figs. 4, C, 5, D). As a matter of fact, the anterior part of the gutter has its cavity directed laterally inwards towards the opening of the oval gland, but the cavity of the remaining portion faces upwards. This different orientation of the cavity of the gutter is due to the fact that the supporting shaft in this region is slightly twisted.

In the posterior quarter of the gutter, the cavity is rather shallow (fig. 5, D), but it narrows and deepens gradually backwards towards the base of the ejaculatory sac (fig. 5, E, F), from the mid-dorsal surface of which a fold extends into the cavity of the gutter.

The outer margin of the gutter is thin and curved slightly downwards (figs. 4, C; 5, B, D, E). It lies closely adjacent to the lateral wall of the ejaculatory sac, where the epithelium becomes very low.

The inner margin on the other hand is rather thick and divided into leaf-like processes which appear in the transverse section (fig. 5, B, C) as finger-like in form. These marginal processes are inserted between the epithelial folds of the ejaculatory sac. In the last quarter of the gutter the inner margin becomes much thickened and its leaf-like processes pronounced (figs. 4, C; 5, D). At the base of the gutter the inner margin assumes the form of a thick, hard plate which is mostly smooth (fig. 5, E).

The gutter gives out anteriorly a *process* which runs transversely and ventrally towards the male genital opening in the common chamber (figs. 4, A; 5, A). This anterior process consists of a thick axis to which is attached a broad flap from the inner side and a narrow one from the outer side. It is suspected that these flaps form a tubular organ for transmitting the sperms from the gutter into the vagina of the female during the process of copulation.

From the base of the gutter are produced several processes of different

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FIG. 5 (plate). A, transverse section of the common chamber showing the two anterior processes of the supporting shafts (aps), the mid-dorsal fold (fo), and the ventral annex glands (vag).

B, transverse section of the ejaculatory organ and its main glands, namely, the cylindrical (cg) and vesicula seminalis, to show the form of the supporting shaft (ss) and the ganglionic mass (gn) at the base of the lumen of the cylindrical gland.

C, portion of a transverse section of the supporting shaft to show the form of its inner margin.

D, transverse section of the ejaculatory organ, at the beginning of the last quarter of the same organ, to show the form of the supporting shaft (ss) and its inner margin.

E, transverse section of the ejaculatory organ, towards the base, to show the oblique vertical process (vp), and the form of the distal portion of the flagellum (dfg).

F, transverse section of the ejaculatory organ at the base, to show the longitudinal process of the shaft (lp) and the form of the distal portion of the flagellum (dfg).

G, transverse section of the ejaculatory organ, further backwards, to show the inner process (ip), the superior outer (so), and inferior outer (io) processes and the distal portion of the flagellum (dfg).

H, transverse section of the flagellum to show the blade (bl) of the posterior process of the shaft in the proximal region of the flagellum (pfg).

I, longitudinal sections of the testis (te) and hepato-pancreas (hp), to show the spermatogonia (spg), spermatocytes (spc), spermatids (spd), and spermatozoa (spz).

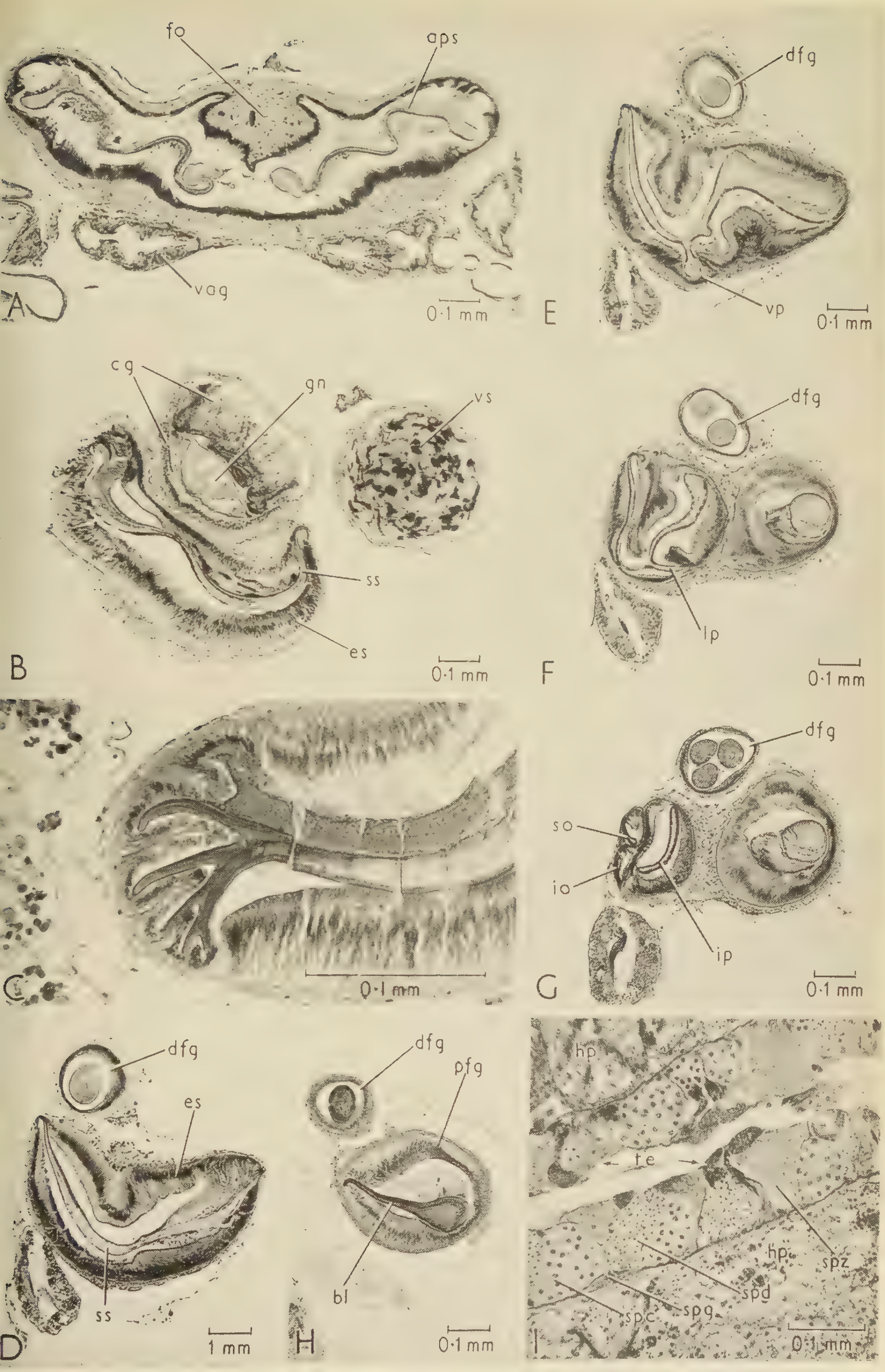

FIG. 5

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form and size (figs. 1, B; 4, A, B). To describe these processes it is found advisable to give them new names according to their mode of orientation. They will be called the oblique vertical, the longitudinal, the outer superior, the outer inferior, the inner, and the posterior processes.

The oblique vertical process arises from the ventral surface of the gutter, at the middle of its base. It extends obliquely downwards in the form of a solid finger-like process and its tip is inserted into the ventral epithelium of the ejaculatory sac (fig. 5, E, vp).

The longitudinal process is in the form of a knife-blade which runs longitudinally backwards. It is attached from its straight edge to the ventral surface of the base of the gutter and lies horizontally with its broad surface pointing upwards (fig. 5, F, lp). It is external to the oblique vertical process and, like the latter, is inserted into an elongated epithelial pocket of the ejaculatory sac.

Further backwards the posterior end of the gutter gives off two outer processes, namely, the superior and inferior outer processes. Both processes are leaf-like, curved, and pointed at their extreme ends. They are inserted by their tips into pocket-like protrusions of the epithelium of the ejaculatory sac (fig. 5, G). The superior outer process is divided into two unequal and narrow processes (fig. 4, B).

From the inner wall of the gutter, at the base, there extends obliquely backwards an inner process, which is the largest and most prominent of all the basal processes. It is curved and pointed. Its ventral surface is supported by a longitudinal thickening and its tip is inserted into a large epithelial protrusion of the wall of the ejaculatory sac (fig. 5, G, ip).

The posterior process extends backwards into the lumen of the flagellum. In the proximal portion of the latter it is provided with a fin-like, transparent blade (figs. 4, A, B; 5, H). In the distal portion of the flagellum it is round in cross-section. Sometimes this portion becomes twisted and in this case appears in transverse section as though made of two or three spherical bodies (fig. 5, F, G).

None of these processes, except the posterior and inner ones, has ever been recorded by any of the previous authors in any scorpion. The account of the posterior and inner processes was incomplete. In *Scorpio occitanus* Dufour (1856) pointed out that there is a sword-like process which corresponds to the inner process of the present species. In *Isometrus maculatus* Pavlovsky (1917) found only two projections, which he referred to as the teeth (*dents*).

Pavlovsky (1921) denied the chitinous nature of the posterior process of the supporting shaft in *Scorpio maurus* and thus he said: 'Elle n'est pas constituée par la chitine car au dire des investigateurs elle ne se dissout pas dans la potasse.'

In the present species, it is found that the whole of the supporting shaft does not dissolve in caustic potash, but it stains with chlorazol black (Cannon, 1937, 1941).

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REFERENCES

Abd-el-Wahab, A., 1952. Proc. Egyptian Acad. Sci., **7**, 75.
Birula, S., 1910. Hor. Soc. Entom. Ross., **39**, 115.
Cannon, H. G., 1937. Nature, **139**, 549.
—— 1941. J. roy. micr. Soc., **61**, 88.
Dufour, L., 1856. Academie des Sciences, Savants Etrangers, **14**, 1.
Pavlovsky, E., 1915. C. r. Soc. biol., **78**, 633.
—— 1917. Ibid., **80**, 502.
—— 1921. Bull. Soc. Hist. Nat. Algér., **12**, 194.
Sato, I., 1940. J. Sci. Hirosima Unit. Zool., **8**, 1.
Sokolow, I., 1913. Arch. Zellforsch., **9**, 399.

The Post-embryonic Development of the Tracheal System in *Drosophila melanogaster*

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SUMMARY

The fate of the tracheal system is traced from the first larval instar to the adult stage. The basic larval pattern conforms to that shown for other Diptera Cyclorrhapha (Whitten, 1955), and is identical in all three instars.

According to previous accounts the adult system directly replaces the larval: the larval system is partly shed, partly histolysed, and the adult system arises from imaginal cell clusters independently of the preceding larval system.

In contrast, it is shown here that in the cephalic, thoracic, and anterior abdominal region there is a definite continuity in the tracheal system, from larval, through pupal to the adult stage, whereas in the posterior abdominal region the larval system is histolysed, and the adult system is independent of it in origin. Moreover, in the pupal stage this region is tracheated by tracheae arising from the anterior abdominal region and belonging to a distinct pupal system.

Moulting of the tracheal linings is complete at the first and second larval ecdyses, but incomplete at the third larval–pupal and pupal–adult ecdyses. In consequence, in both pupal and adult systems there are tracheae which are secreted around pre-existing tracheae, others formed as new 'branch' tracheae, and those which have been carried over from the previous instar. In the adult the newly formed tracheae of the posterior abdominal region fall into a fourth category. Most of the adult thoracic air sacs correspond to new 'branch' tracheae of other instars.

The pre-pupal moult and instar are discussed with reference to the tracheal system and tentative suggestions are made concerning the true nature of the pre-pupal cuticle. There is no pre-pupal tracheal system.

Events traced for *Drosophila* would seem to be general for Cyclorrhapha, both Acalypterae and Calypterae. The separate fates of the anterior and posterior abdominal systems, in contrast with the straightforward development in Diptera Nematocera, would appear to mark a distinct step in the evolution of the system in Diptera.

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INTRODUCTION

ALTHOUGH the tracheal system of insects has been much studied knowledge of the development of the system is scanty and contains many contradictions. Older workers came to one of two conclusions concerning the fate of the system during metamorphosis in holometabolous insects either that the tracheal system is entirely destroyed during the pupal stage so that the adult system is independent of the pre-existing larval system; o else that small parts of the main tracheae of the adult system are formed around stumps of previous larval tracheae (Peréz, 1910), the rest of the larva system being destroyed by 'histolysis', and the remainder of the adult system arising independently of pre-existing larval tracheae. Keister's (1948) recen results on the development of the tracheal system in *Sciara coprophil* (Diptera Nematocera) favour Palmén's (1877) belief that the same trachea epithelium persists right through to the adult stage, rather than Peréz's (1910 conception of the origin of the adult system from nests of imaginal cells o the larval tracheal stumps.

The present investigation in *Drosophila melanogaster* Meigen (Diptera Cyclorrhapha) is an attempt to determine the detailed topography of th tracheal system in each of the stages from the first larval instar to the adul stage, and to investigate the relationship existing between them. The mai object has been to determine whether there is a definite continuity in th system from larva to adult, or whether, as concluded by Robertson (1936) an Bodenstein (1950), the adult system directly replaces and is independent o the larval system, in which case there is no distinct functional pupal system

The development of the tracheal system is a more difficult problem in a member of the Diptera Cyclorrhapha than is the same problem in th Nematocera, of which *Sciara coprophila* is a member (Keister, 1948). In th Nematocera there are four larval instars, a pupal instar, and lastly the adul stage. Each instar possesses its own tracheal system, and by a relatively simpl process the cuticle and tracheal system of any instar is replaced by that of th subsequent instar, the tracheal system of the former being divided into it component metameres and removed through the respective pairs of spiracles The new tracheal intima is secreted around the pre-existing one. In this wa the fourth larval system is replaced by the pupal, and this in turn by the adult In the Cyclorrhapha the problem is complicated by the developmental processes occurring between the last larval instar and adult stage. Instead o four, there are three larval instars, and the last (third) larval cuticle is retained as the so-called 'puparium'. Existing evidence of the processes occurring subsequently to this is contradictory. Fraenkel (1938) observed in *Calliphora erythrocephala* that the abdomen only of the pupa is invested in a thin membrane lying between pupal cuticle and the puparium. Previously to thi Snodgrass (1924) noted a thin membrane completely investing the pupa o *Rhagoletis*, and he suggested that it is a pre-pupal cuticle which correspond to the cuticle of a suppressed fourth instar larva. This would bring th development more in line with that found in the Diptera Nematocera, where there are four larval instars. A study of the tracheal system must necessarily involve a consideration of the general cuticle with which it is continuous. Robertson (1936) for *Drosophila* reports the presence of a pre-pupal cuticle completely investing the larva, but does not discuss it in connexion with the tracheal system.

Details of the moulting of the larger and finer tracheal branches in the different developmental stages of *Drosophila melanogaster* are also considered. Keister (1948) showed that in *Sciara coprophila*, whereas shedding of both tracheal and tracheolar intima is complete at each of the inter-larval ecdyses, at the pre-pupal and pre-adult ecdyses certain of the pre-existing ultimate branches are retained and not shed, so that the final adult system possesses a number of pupal and even preceding larval elements. On the other hand, Wigglesworth (1954) has shown for *Rhodnius prolixus* (Hemiptera) that at each ecdysis the lining of the tracheole is never shed with the cuticle, although the lining of the trachea is removed.

Materials and Methods

All stages were studied either in the living state or after injection with cobalt sulphide (Wigglesworth, 1950). After injection, specimens were dissected or sectioned. The living stages were investigated by dissection under a binocular dissecting microscope, while the details of moulting were followed exclusively by phase-contrast microscopy.

Whereas the events taking place in the tracheal system at, before, and after the inter-larval ecdyses can be seen through the transparent body-wall, opacity of the puparium makes such a study of the subsequent stages difficult, and in the later stages almost impossible. Although individual specimens are only 3 mm in length, the sequence of events subsequent to puparium formation is not simultaneously observable in all the tracheae. Therefore, the following account is of a composite picture obtained from several hundred whole and dissected, living and injected specimens.

The work was begun in the Zoological Laboratory, Cambridge, and has been continued at Queen Mary College (University of London).

The Larval System

The tracheal system of the larva is essentially similar in all three instars, the chief difference being the presence of an increasing number of smaller tracheae and tracheoles in the successive stages. The pattern formed by the main tracheae is identical with that seen and described by Whitten (1955) in other Diptera Cyclorrpapha. Each of the main tracheae can, therefore, be homologized with those described for other Diptera, and the terms used in the following description correspond to those given in the above paper, rather than those given in the descriptive accounts by Ruhle (1932) and Haskins and Enzmann (1937). The larval system is redescribed in detail as both of the latter descriptions omit several tracheae which form an essential part of th basic tracheal framework; this must be determined in detail before the subsequent development can be traced.

The first instar system is metapneustic, while the second and third ar amphipneustic. The spiracular tracheae of the non-functional spiracles two to nine are collapsed to form solid spiracular cords.

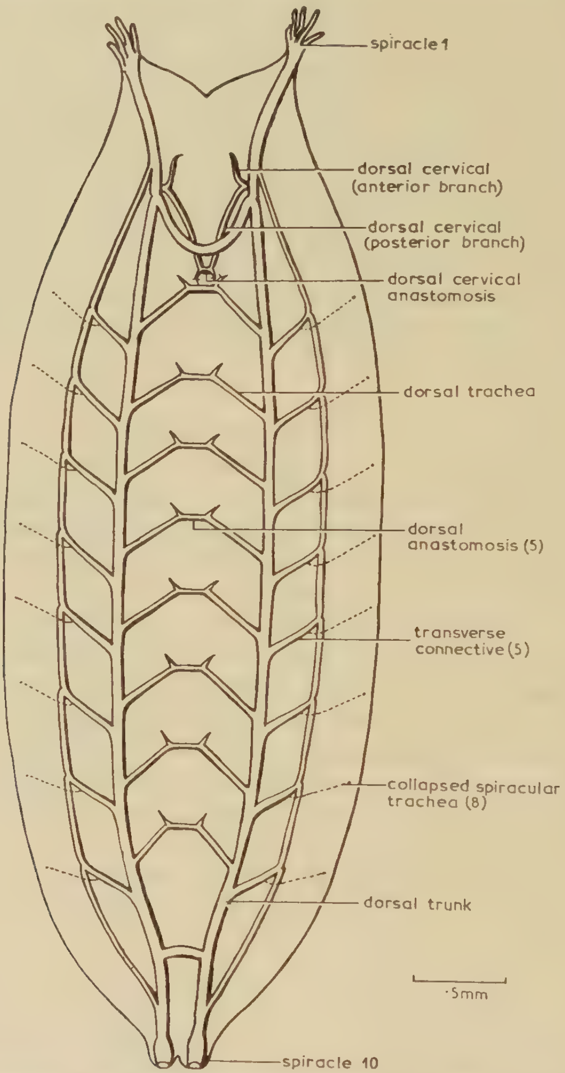
spiracle 1
dorsal cervical (anterior branch)
dorsal cervical (posterior branch)
dorsal cervical anastomosis
dorsal trachea
dorsal anastomosis (5)
transverse connective (5)
collapsed spiracular trachea (8)
dorsal trunk
·5mm
spiracle 10

FIG. 1, A. *Drosophila*: the larval tracheal system. Dorsal view. Semi-diagrammatic.

In common with other Diptera Cyclorrhapha, there are a pair of dorsal and a pair of lateral longitudinal trunks, joined on each side by eight transverse connectives which give off the spiracular threads of spiracles two to nine. Connecting the two dorsal trunks are ten dorsal anastomoses arranged as in fig. 1, A.

Posterior to the anterior junctions of the lateral and dorsal trunks, a dorsal cervical trachea arises from each dorsal trunk. Shortly after its origin each

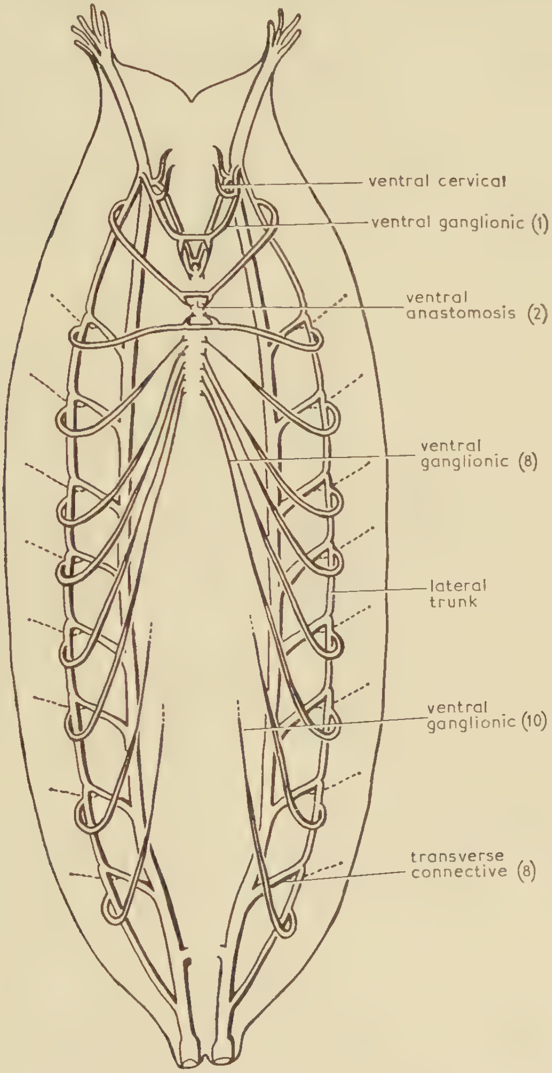

FIG. 1, B. *Drosophila*: the larval tracheal system. Ventral view. Semi-diagrammatic.

divides. At the level of the supraoesophageal ganglia each posterior branch gives off a trachea to the ganglion and then unites with its fellow in a short median anastomosis corresponding to the dorsal cervical anastomosis of other Diptera (Whitten, 1955). This is omitted from the description by Haskins and Enzmann (1937).

Each ventral cervical trachea, before passing anteriorly, gives rise to medianly directed trachea; this is one of the first pair of ventral ganglioni tracheae. It gives off a branch to the central nervous system, and almos immediately anastomoses mid-ventrally with its fellow to form the first ventra anastomosis. Only the first three pairs of ventral ganglionic tracheae forr mid-ventral anastomoses. These tracheate areas of the nerve mass corresponding to the pro-, meso-, and meta-thoracic ganglia of the Diptera Nematocera

The last two pairs of ventral ganglionic tracheae, instead of traversing th abdomen to tracheate the posterior region of the ventral nerve mass, terminat abruptly without reaching the nerve mass. This is obviously a variation from the normal condition seen in the Diptera Cyclorrhapha; possibly othe species of *Drosophila* or even different strains of *melanogaster* are normal in this respect. Haskins and Enzmann (1937) described the same condition in their material.

The Pupal System

The functional pupal tracheal system differs very greatly from the larval The metameric tracheal pattern so characteristic of the system in the larva is not immediately evident. However, on closer investigation, such a pattern can be made out in the thoracic and anterior abdominal regions.

The only functional spiracles at this stage are the first thoracic pair which are well developed, biperforate, and lie at the anterior angles of the prothorax.

The head is tracheated by numerous ramifications of two pairs of tracheae, both of which arise from the region of the functional 'prothoracic' spiracles. One pair enters the head ventrally to the other and tracheates the ventral regions of the head, including the developing proboscis. The two dorsal tracheae, almost immediately on entering the head, are connected by a transverse anastomosis. The dorsal tracheae send numerous branches to the developing eyes and to the brain.

The most striking differences from the larval tracheal system are in the abdominal region where, posteriorly, there is no evidence of a metamerically arranged pattern of tracheae connected with either non-functional or functional spiracles. Instead, the whole of this region is tracheated by the numerous ramifications of two pairs of tracheae, arising from the anterior abdominal region.

The thoracic and anterior abdominal tracheae are best considered together. The spiracular trachea opening at the 'prothoracic' spiracle is very broad and continues posteriorly as a longitudinal dorsal tracheal trunk. From it, shortly after its origin from the spiracle, a trachea of much narrower diameter arises, which also passes posteriorly as a lateral longitudinal trunk. Both continue as far as the anterior abdominal region.

The two broad longitudinal trunks are connected dorsally by five dorsal anastomoses. All, except the first, are of very narrow diameter, and obscured beneath opaque pupal cells, being visible only by micro-dissection. The second differs from the three succeeding ones by giving rise to numerous racheae and tracheoles which supply the developing thoracic flight muscles. The first of these anastomoses is very much broader in diameter and unites he two dorsal trunks posteriorly to the origin of the lateral trunks.

The two pairs of trunks of each side are connected by four tracheae. The

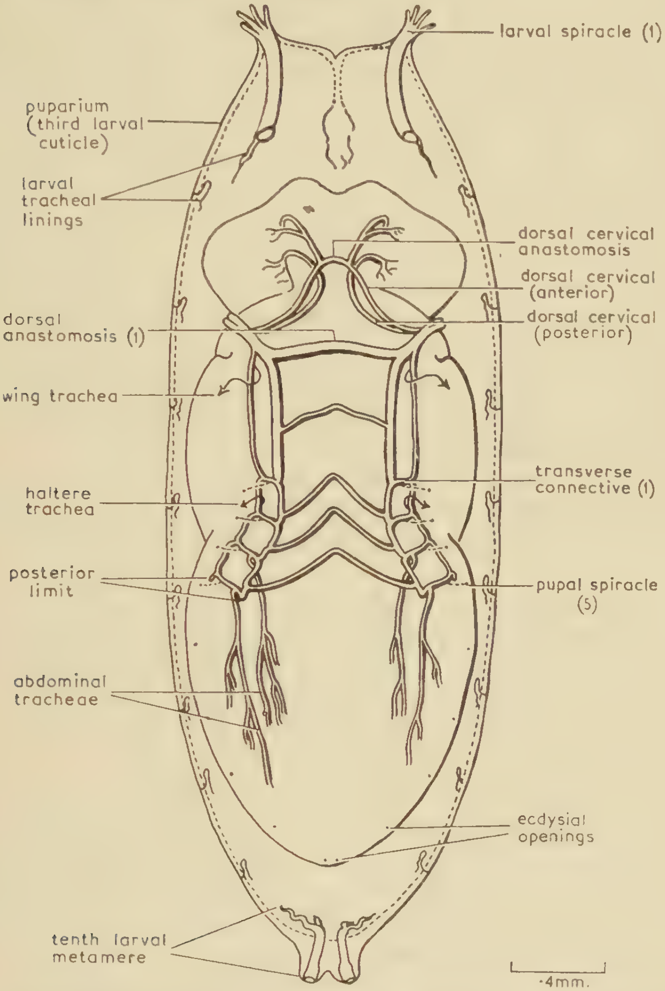

FIG. 2. *Drosophila*: the pupal tracheal system. Dorsal view. Semi-diagrammatic.

rst arises from the dorsal trunk posteriorly to the origin of the second dorsal nastomosis. The succeeding connectives arise between the origins of successive dorsal anastomoses. This arrangement immediately recalls an identical rrangement between the anterior dorsal anastomoses and the transverse onnectives of the larval system.

From each of the lateral trunks, immediately posterior to their origin, a rachea arises which passes ventrally. It first gives off a trachea to the first

pair of legs, then a trachea to the nervous system, and finally unites with i
fellow in the mid-ventral line. Posteriorly to the origin of the first ganglio
tracheae each lateral trunk gives rise to a trachea which, passing medi
ventrally, first gives off a trachea to the nervous system and then unites wi
its fellow in the mid-line. The second pair of leg tracheae arises from t
lateral trunk separately and posteriorly to the origin of the second pair
ventrally anastomosing tracheae. A third pair of ventrally anastomosi
tracheae and the third pair of leg tracheae arise separately and in a correspon
ing position to the second pairs, but between the origins of the first a
second transverse connectives. The relative positions of these ventral trache
are clearly identical with those of the first three ventral ganglionic trache
of the larval system; they similarly unite in the mid-line to form three ventr
anastomoses.

A trachea to each wing arises from the lateral longitudinal trunks i
mediately after their origin anteriorly. Each bifurcates near the wing ba
both branches entering the wing. No corresponding posterior wing trach
has been observed either in dissection or after injection. In the case of t
haltere, a single trachea arises from the lateral trunk just anteriorly to t
origin of the second transverse connective; no corresponding anterior trach
appears to reach or enter the haltere.

The spiracular tracheae associated with the four pairs of transverse co
nectives appear collapsed and the corresponding spiracles inconspicuous a
non-functional.

The two pairs of very much-branched tracheae which ramify throughout t
posterior abdominal region arise from the third and fourth transverse co
nectives at the origin of these from the dorsal trunks. Posteriorly to the origi
of the fourth connectives, the broad dorsal trunks end blindly in a chara
teristic and curious way.

THE ADULT SYSTEM

The adult tracheal system bears even less resemblance to that of the pr
ceding pupal and larval stages than does the pupal to the larval system. Mill
(1950), in describing the adult system, simply remarks on the enormo
changes which have taken place in the head and thoracic region, accompan
ing evolution of air sacs. According to him the basic metameric pattern
implying the serially repeated pattern characteristic of the insect trache
system in general—is clearly evident only in the mid-abdominal region. H
does not attempt to compare the system with that found in the larva, n
could he have compared his description with those of other Diptera, for h
is one of the rare accurate descriptions in print of the tracheal system of
adult dipteran (see Whitten, 1953).

For the purpose of tracing the development of the system through to t
adult, it is necessary to redescribe the adult system, concentrating on the ba
pattern underlying the complex arrangement of air sacs in the head a
thorax. Consequently, Miller's descriptive terms for the different air sacs w

ot be used consistently, but only where they help in describing the detailed tructure overlying the fundamental pattern.

In the adult there are nine pairs of functional spiracles. They occupy posiions corresponding to the first nine pairs of larval spiracles, which are, ccording to Keilin (1944) intersegmental in position.

The head (figs. 3, 9) contains a large number of air sacs which give off umerous tracheae and tracheoles supplying the eyes, brain, and other strucures. According to Miller (1950) the distal air sacs all arise from a pair of entral and a pair of dorsal sacs which have diverged from a single pair of acs entering the head from the region of the 'pro-thoracic' spiracles. It vould, however, appear that the paired dorsal and ventral sacs actually nter the head separately, as two pairs of sacs, those of the two sides being very losely apposed and therefore appearing to be single. Each of the dorsal sacs irst gives off a branch to the brain; this is narrow in diameter and tracheate in orm. Secondly, a trachea is given off laterally which expands into the post-cular air sac of Miller (1950); this passes antero-ventrally and tracheates the yes. This is followed by a sac-like branch arising medially which anastomoses vith its fellow mid-dorsally. Opposite the origin of this, there arises laterally a ranch which tracheates the brain. The main dorsal vessels finally expand nto the paired dorsal sacs. The paired ventral sacs each give off first a very arge frontal sac and then continue as the post-genal air sacs which ultimately ass into a trachea that extends the length of the proboscis, supplying branches o the various structures.

In the abdomen the system is clearly composed of tracheae constituting a efinite pattern. Passing from the first pair of spiracles to the posterior end re two pairs of longitudinal trunks. The first pair, situated more dorsally, egins anteriorly as a continuation backwards of the first thoracic spiracular rachea. It is very broad in diameter and generally sac-like until reaching the egion of the fifth pair of spiracles. Here it suddenly becomes narrow and coninues in its more tracheate form as far as the posterior end of the abdomen. n the thoracic region, tracheae and tracheoles arising from it pass mainly to he dorsal median indirect flight muscles. The more laterally placed trunk is oined to the dorsal trunk immediately behind the first thoracic spiracle. In he thoracic region it is hardly recognizable as a single longitudinal element, eing expanded into a series of air sacs. At the level of the third pair of piracles it becomes more tracheate and extends in this form to the region of he ninth pair of spiracles. In the thoracic region small branches arise from he sac-like dilations of the lateral trunks to tracheate mainly the more supericial tergo-sternal flight muscles.

The dorsal and lateral trunks of each side are connected, besides their juncions anteriorly and posteriorly, in seven places. In the abdominal region they re connected by long transverse connectives; in the region of the second air of spiracles the connectives are much shorter and stouter.

The two broad dorsal trunks are connected by a regularly placed series of ine dorsal anastomoses. The first, which is much broader and more sac-like than the rest, and the second, which is very narrow, occur between the regi of the first thoracic spiracles and the first pair of connectives, at the level the second pair of spiracles. The subsequent anastomoses arise between th origins of successive transverse connectives and the ninth pair of spiracles.

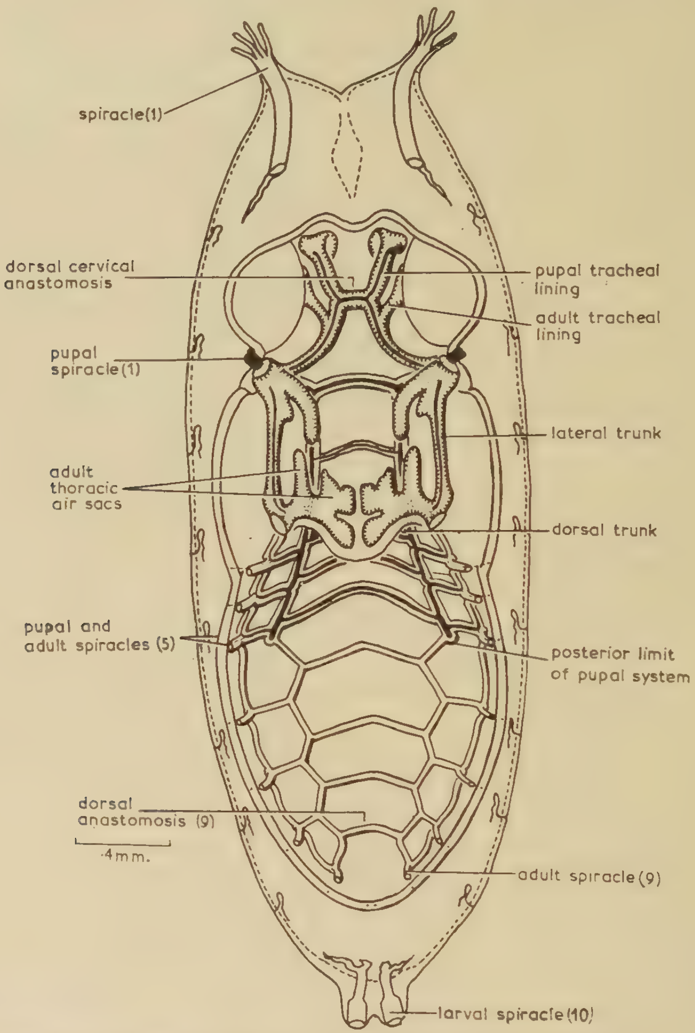

FIG. 3. *Drosophila*: the adult tracheal system showing its relation to the preceding pup system. Dorsal view. Semi-diagrammatic.

The third to ninth pairs of spiracular tracheae arise from the transvers connectives at the ventral end near their junction with the lateral trunks.

Other tracheae in the abdominal region will not be considered here as the hardly constitute part of the fundamental pattern. It is significant tha posteriorly a tenth pair of spiracles appears to be absent. In fact, a tent tracheal metamere—as found in the larva—appears to be completely absent, so reducing the total complement of spiracles and dorsal anastomoses from ten to nine.

In the thorax the basic framework is obscured by the many large air sacs (figs. 3, 10). The three leg tracheae arise in positions corresponding exactly to those in the pupa. The second pair arises directly from the lateral trunks anteriorly to the second pair of spiracles. The third pair similarly arises from the lateral trunks anteriorly to the third pair of spiracles. The first pair, however, arises from a pair of branches tracheating the nervous system. These correspond in position and origin to the first ventral ganglionic tracheae of the pupa and larva and likewise form a mid-ventral anastomosis. The second and third pairs of ventral ganglionic tracheae similarly arise directly from the lateral trunks anteriorly to the origins of the respective second and third leg tracheae. The second ganglionic trachea is given off by the expanded portion of the lateral trunk, posteriorly to the first thoracic spiracle. The third anastomosis is distinctly sac-like, but the tracheae arising from it to pass to the nervous system are tracheate.

The wing and haltere tracheae also arise in positions corresponding to those in the pupa. They are narrow in diameter, except that the base of the wing trachea arises from a small air sac. Neither the wing nor haltere tracheae are described by Miller (1950). From Chapman's (1918) review of the wing tracheae in insects, two pairs of both wing and haltere tracheae might have been expected. In *Drosophila* they are very difficult to follow, but certainly neither in the living nor injected adult has a second trachea been found to penetrate either the wing or haltere.

The First Larval Ecdysis

The tracheal lining of the second instar larval system is secreted around that of the first, extending even into the finest tracheae and tracheoles. The result is that the larval lining of the first instar is completely replaced by that of the second. In addition, a number of new secondary 'branch' tracheae are formed. Quite a while before ecdysis occurs taenidia appear in the newly formed lining. Immediately before the moult the old lining breaks into its ten tracheal metameres, as demonstrated by Keilin (1944) for dipteran larvae in general. The breaks in the longitudinal trunks occur posteriorly to the origin of the respective transverse connectives and in the mid-line in the dorsal and ventral anastomoses and dorsal cervical anastomosis.

At ecdysis, the tracheal metameres, being attached to the cuticle at their respective spiracles, are withdrawn along with them. In this way the cervical tracheae, first dorsal anastomosis, and part of the longitudinal trunks together with their branches are removed with the first pair of spiracles; similarly, the second dorsal anastomosis, first transverse connective, and relative parts of the trunks and their tracheae are withdrawn with the second pair of spiracles, and so on.

After withdrawal of the old intima, the second instar system rapidly fills with air and the second to ninth spiracular tracheae collapse, while the spiracular tracheae of the first and last pairs of spiracles remain open. The places through which the old system was removed close up and remain as ecdysial scars, which are inconspicuous even in the larger first and last pairs of spiracles.

THE SECOND LARVAL ECDYSIS

The details of this ecdysis are very similar to the events recorded for the first larval ecdysis. In the cases of the tracheae in which the moulting process has been followed in detail, moulting is complete; even the intima of the tracheoles is withdrawn and replaced by a new third instar tracheal lining. Besides the addition of new 'branch' tracheae the main difference lies in the formation of the more elaborate last pair of spiracles and of the finger-like anterior spiracles. The spiracular tracheae of spiracles two to nine again collapse to form solid cords.

THE LARVAL–PUPAL ECDYSIS

Prior to pupation, the fully grown third instar larva ceases to feed and extends its anterior and posterior spiracles; the larval cuticle gradually becomes hardened to form the characteristic puparium with its operculum demarcated anteriorly. Darkening occurs gradually and for some time the puparium remains sufficiently light in colour for the internal tracheal system to be observed through the puparium wall, at least in the dorsal region of the anterior end. Right from the beginning of puparium formation, changes occurring in the ventral and in the more posterior region are more difficult to see.

Through the puparium wall the pupal first thoracic spiracles can be seen developing median to the base of the larval first thoracic spiracular tracheae (figs. 4, 6). Later, the tracheal lining within the spiracles is secreted and is seen to be continuous with the new pupal lining which is secreted at the same time; this is laid down around the existing larval lining and not independently of it. The new pupal tracheal lining is secreted around the first five dorsal anastomoses, the first four transverse connectives, and the longitudinal trunks *only* as far as the region immediately posterior to the origin of the fourth pair of transverse connectives. At this point, larval and pupal tracheal linings are confluent. Posteriorly to this, no pupal lining—discernible under an oil immersion objective—is secreted by the tracheal epithelium. This striking state of affairs was re-confirmed many times and is substantiated by subsequent events.

Not all of the tracheae in the anterior region are surrounded by a newly secreted pupal tracheal lining; in this respect the process is essentially different from that occurring at the inter-larval ecdyses where the lining, even in the finest tracheae and tracheoles, appears to be replaced. Examples of tracheae in which the larval intima is retained in the pupal stage include the ganglionic tracheae arising from the ventral anastomoses (fig. 5, D, E), and the tracheae arising from the dorsal anastomoses (fig. 5, A). In contrast, at the inter-larval ecdyses both the tracheae and their tracheoles are surrounded by the newly secreted intima and moulting is complete.

The processes occurring at this stage differ in another remarkable respect from those taking place at the previous ecdyses. Whereas at the inter-larval ecdyses the production of new 'branch' tracheae and tracheoles is confined to elements of small diameter and of relative unimportance, when the pupal tracheal system is formed a number of large tracheae, giving rise to numerous smaller ramifications, are laid down. However, they are essentially similar to

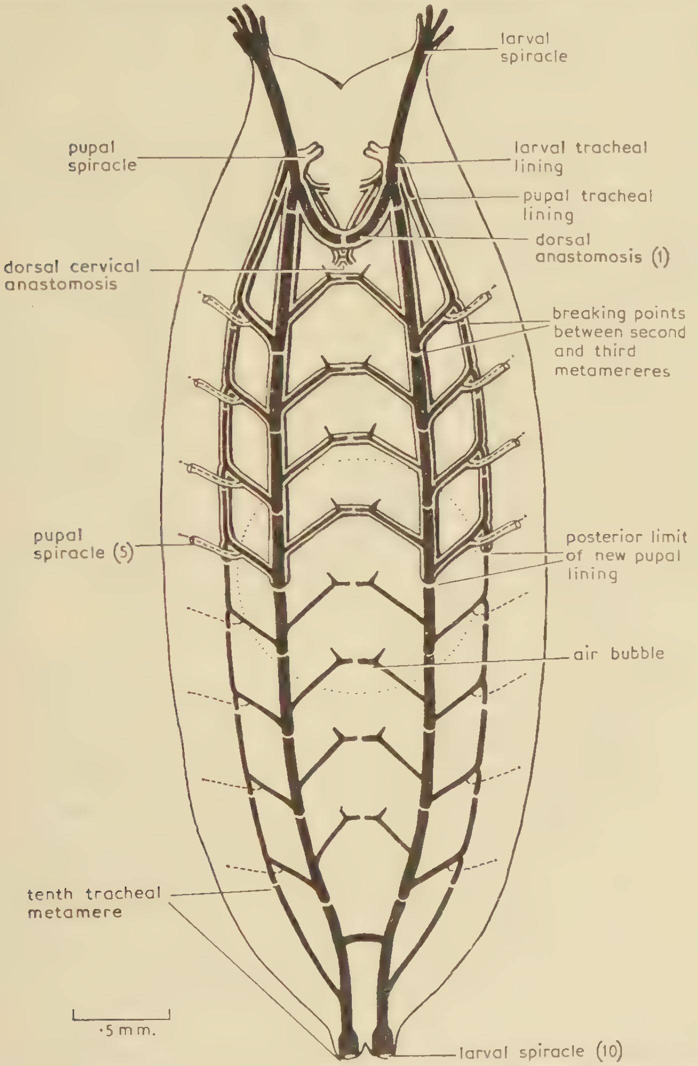

FIG. 4. *Drosophila*: reconstruction of third instar larva shortly before pupation, showing the position of the newly secreted pupal lining relative to the tracheal lining of the preceding larval stage. Dorsal view. Diagrammatic.

the smaller 'branch' tracheae of previous instars in that their lining appea simultaneously to, and is continuous with, that secreted around the neighbouring pre-existing framework of larval tracheae. The most striking of thes exclusively pupal branch-tracheae are (*a*) tracheae arising from the tw branches of the cervical tracheae, which will eventually tracheate the developing head structures including the eyes (fig. 5, B, C), (*b*) the numerous fine tracheae arising from the second dorsal anastomosis which will subsequently tracheate the developing thoracic flight muscles (fig. 5, A), and (*c*) the two pairs of large tracheae arising from the third and fourth transverse connectives immediately after their origin from the dorsal longitudinal trunks (fig. 11, A); these ramifying tracheae will be responsible for the tracheation of the entire posterior abdominal region.

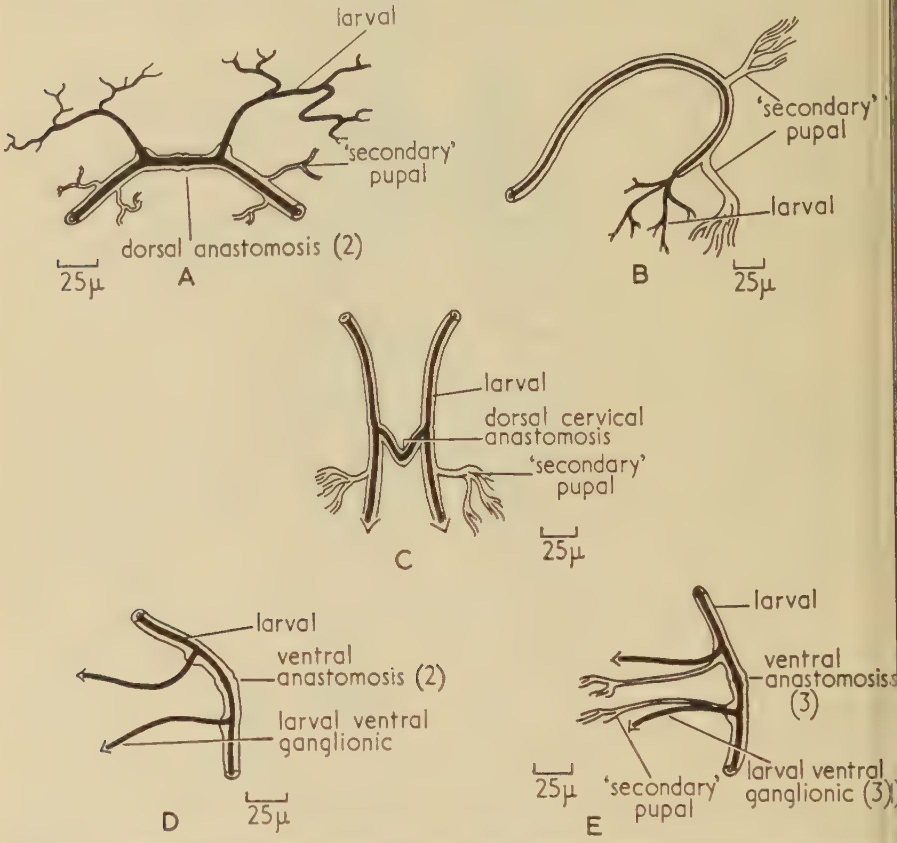

FIG. 5. *Drosophila*: individual tracheae of the third instar larva shortly before pupation showing the positions of the existing larval relative to the recently secreted pupal tracheal linings. A, the second dorsal anastomosis. B, the ventral cervical trachea. C, the dorsal cervical trachea. D, the second ventral anastomosis. E, the third ventral anastomosis.

Before withdrawal of the larval tracheal linings, breaks occur in the larval tracheal system dividing it into its ten pairs of tracheal metameres. Withdrawal of the larval linings starts first in the anterior region, extending subsequently to the abdominal metameres. Each pair of metameres is withdrawn through a pair of ecdysial openings in the pupal cuticle. In the case of the first pair of tracheal metameres (fig. 6) breaks occur mid-dorsally in the dorsal cervical anastomosis and first dorsal anastomosis, and in dorsal and lateral trunks in the region immediately posterior to the first dorsal anastomosis. Simultaneously with the severing and withdrawal of these larval tracheae associated with the first pair of spiracles, internal cell movements are set up, and the head becomes evaginated. The two pairs of posteriorly directed dorsal cervical tracheae and the cervical anastomosis are swept forward in the general stream and come to lie inside the everted head (fig. 8, B). The first dorsal anastomosis, initially superficial, comes to lie in a deeper position, almost completely obscured dorsally by pupal cells. Breaks in the cervical tracheae occur at the junction of larval and pupal linings so that the ultimate tracheae are larval, the main tracheae are pupal formations around pre-existing larval, while the much-divided branches are new pupal structures having no precursors in the preceding larval stage.

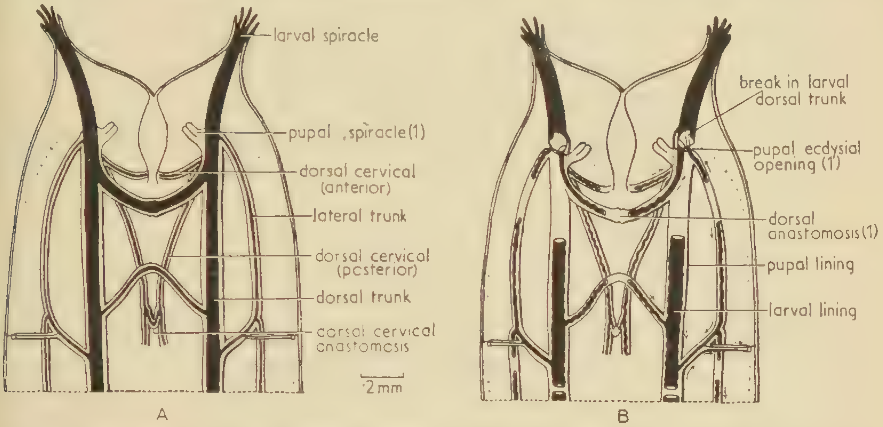
larval spiracle
pupal spiracle(1)
dorsal cervical (anterior)
lateral trunk
dorsal cervical (posterior)
dorsal trunk
dorsal cervical anastomosis
·2 mm
A
break in larval dorsal trunk
pupal ecdysial opening (1)
dorsal anastomosis(1)
pupal lining
larval lining
B

FIG. 6. *Drosophila*: the larval tracheal system. A, before ecdysis of the larval tracheal linings. B, at ecdysis of the larval tracheal linings. Anterior end in dorsal view. Semi-diagrammatic.

As the tracheae attached to the larval first spiracles are withdrawn, they become collapsed, whereas the spiracular trachea itself always remains air-filled and rigid. The 'hole' (fig. 6, B) in the tracheal lining—at the extremity of the spiracular trachea—simply represents, morphologically, the breaking point in the dorsal longitudinal trunk. The portion of the trunk posteriorly to this belongs to the second metamere; the latter, with its associated tracheae, is withdrawn along with the second pair of spiracles.

Withdrawal of the larva away from the puparium wall is followed by contractions of the abdomen and secretion of the thin 'pre-pupal' membrane. Simultaneously with these contractions a large air bubble (fig. 4) lying within the larval abdomen is released, and the air rapidly spreads between the pupal cuticle and the newly secreted 'membrane'. The more posterior larval tracheal metameres are simultaneously withdrawn and comes to lie on the inner surface of the membrane. The spiracular threads of the second to ninth spiracles remain attached to the non-functional spiracles in the wall of the puparium. The threads themselves traverse the space between the puparium and the thin membrane, pass through the latter, and open into the collapsed tracheae which lie on its inner surface.

If a pupa is removed from the puparium immediately after the withdrawal of the larval tracheae, air bubbles are to be seen attached to the abdominal ecdysial openings; this suggests that the bubble of air lying within the larva in some way becomes transferred to the outside of the pupa by way of these ecdysial openings. This may be supplementary to its possible escape more posteriorly during simultaneous removal of the larval proctodaeal lining. As noted by Robertson (1936) the bubble within the larva rapidly decreases in size at the time of, and subsequently to, the appearance and spreading of air between the pupal cuticle and pre-pupal membrane.

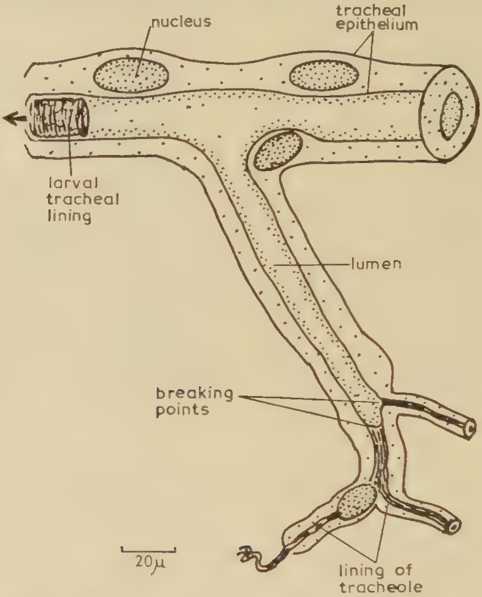

FIG. 7. *Calliphora*: a trachea from the posterior abdominal region of a third instar larva during withdrawal of the tracheal linings of the larva.

A pupa dissected immediately after withdrawal of the larval tracheae shows that the tracheal epithelium of the posterior abdominal region is intact and continuous with that of the more anterior region, but there is no corresponding tracheal lining. The breaking point in the dorsal trunks between the removed sixth pair of larval metameres and the new pupal lining of the fifth tracheal metameres can be clearly seen. The open end of the air-filled tube appears to contract either by its own elasticity or by that of the taenidia in its walls: there is no escape of air from the open end nor entry of fluid from the region posterior to it. Dissected pupae of later stages reveal the presence of the more anterior tracheal epithelium only; by this stage the posterior parts of the larval tracheal epithelium have disappeared, and it must be inferred that it has been histolysed. Fig. 7 shows a portion of a trachea of the posterior abdominal region in *Calliphora erythrocephala* immediately after withdrawal of the larval tracheal lining. Here, seen under the highest magnification available, a distinct break occurs in the larval lining between the trachea and tracheole; the lining of the tracheoles is retained and presumably absorbed along with the cells, while the lining of the trachea is withdrawn, leaving behind only the epithelial cells. As the events taking place in *Calliphora* have, in other respects, been found to parallel so closely those in *Drosophila*, the same retention of tracheolar linings probably also occurs in *Drosophila*.

The larval metameres are removed through the usual number of ten pairs of ecdysial openings in the pupal cuticle. These subsequently close to form minute ecdysial scars. In the anterior region the first pair of spiracles remains functional but the spiracular tracheae of the second to fifth appear collapsed and the spiracles non-functional.

THE PUPAL–ADULT ECDYSIS

The basic framework of the adult system is formed by secretion around the linings of the pupal system (figs. 8, C; 3, 9, 10), whilst most of the larger air sacs of the head and thorax correspond to 'secondary' branch tracheae. In some cases this newly secreted adult intima is not significantly greater in diameter than the enclosed pupal intima. However, in the case of the air sacs the epithelium has become withdrawn from the pupal lining to a considerable extent, and the newly secreted adult lining is very much folded and of considerably greater diameter than the enveloped pupal intima (fig. 12). Whatever the peculiar properties of the epithelium which secretes the lining of the more sac-like parts, it is continuous with the lining, giving rise to the more tracheate adult elements. In fact, in the region between first and second pairs of spiracles the tubular longitudinal trunks of the pupa can be seen surrounded by the adult intima, which anteriorly and posteriorly is secreted in sac-like form separated by a distinctly tracheate region (fig. 10).

In many cases, as at the pupal ecdysis, the newly secreted adult intima does not extend into the smaller tracheae and tracheoles, and as a result the pupal tracheae are retained in the adult. This happens in the case of the ganglionic tracheae arising from the thoracic ventral anastomoses, and the wing and haltere tracheae (fig. 8). The air sacs arising from the region of the first and second pairs of 'thoracic' spiracles correspond to the 'branches' of previous stages (fig. 10). Their intima is secreted simultaneously with, and is continuous with, that of the adult intima secreted around the pre-existing pupal trunks. While still within the puparium they also have a broad, collapsed, much-folded appearance.

In the abdomen (figs. 3, 11) the adult intima is secreted in a tracheate form around the pupal tracheae and in the later stages taenidia are clearly seen.

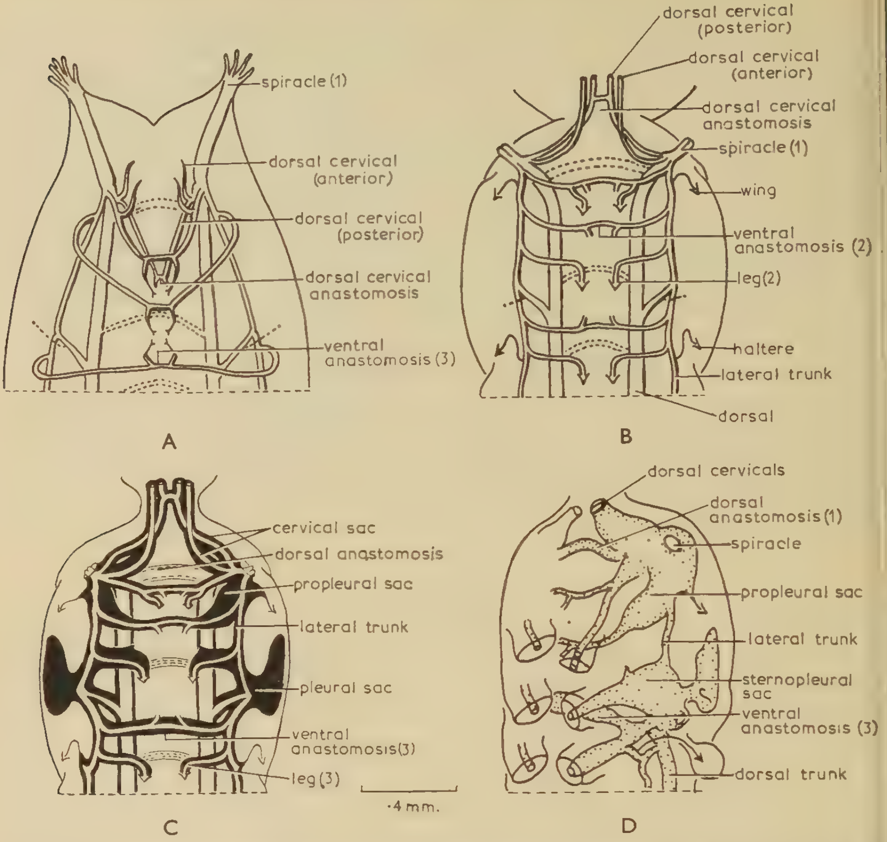

FIG. 8. *Drosophila*: the anterior end of the tracheal system in ventral view A, the larva. B, the pupa. C, the late pupa showing the relationship of the pupal to the newly secreted adul linings. D, the adult. Semi-diagrammatic.

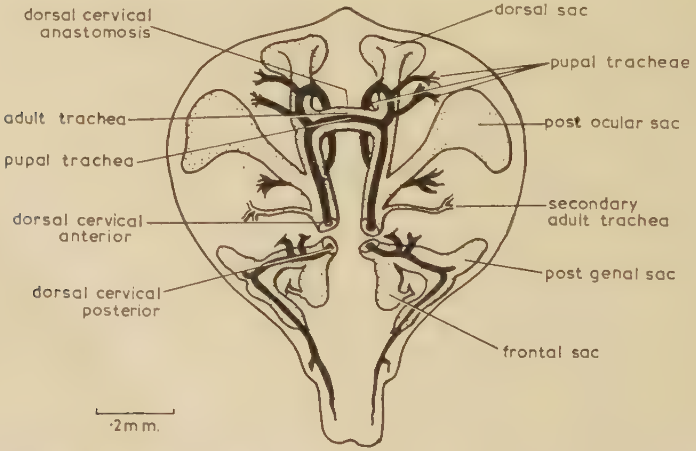

FIG. 9. *Drosophila*: the head of a late pupa in posterior view, showing the relative positions of the pupal and newly secreted adult linings (air sacs shown in expanded air-filled condition).

The tracheal epithelium surrounds the posterior 'broken' end of the pupal trunk, and at the inner posterior angle continues in a medio-posterior direction. It is here much narrower in diameter as also is the adult intima which it secretes: this is probably due to its not surrounding a pre-existing pupal trachea. The main tracheae forming this posterior adult system are the two longitudinal trunks, four dorsal anastomoses, three transverse connectives, and four pairs of spiracles. A tenth tracheal metamere does not appear to be formed.

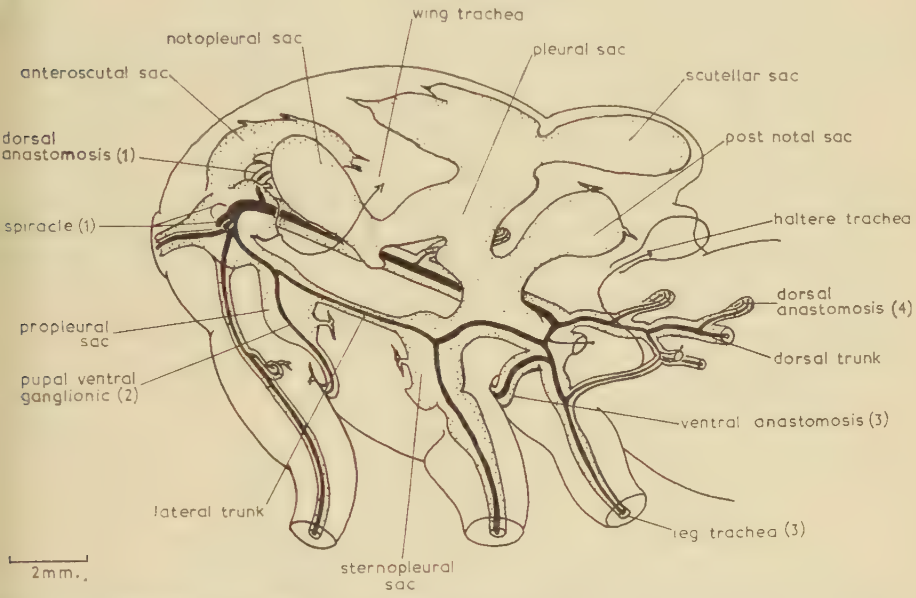

FIG. 10. *Drosophila*: the thorax of a late pupa in left lateral view, showing the relative positions of pupal and newly secreted adult linings (air sacs shown in expanded air-filled condition).

At ecdysis, breaks occur in the pupal intima, dividing the system into the five pairs of tracheal metameres of which it is composed. The breaks occur in positions corresponding to those in the first five pairs of larval tracheal metameres, i.e. in the mid-dorsal and mid-ventral lines, and along the longitudinal trunks in the region posterior to each respective transverse connective.

Fig. 11, C is of the mid-abdominal region of a mature pupa of *Calliphora erythrocephala* (Cyclorrhapha, Calypterae). It shows the relationship of the newly secreted adult tracheal lining to the existing functional pupal system. The relationships are almost identical with those of *Drosophila* (fig. 11, B). The only significant difference is that the lining around the dorsal trunk is sac-like in *Calliphora*. This is particularly interesting in that the presence of a pair of large air sacs in the abdomen is fairly general for Calypterae, whereas they are generally absent in Acalypterae.

Great changes appear to take place in the tracheal epithelium during the pupal stage. The present investigation has been mainly concerned with the

fate of the tracheal lining at the different ecdyses. Although this is closel
linked with the fate of the tracheal epithelium itself, work remains to be don
on the fate of the individual cells constituting the epithelial layer to determine
the extent of reorganization, replacement, and histolysis which occurs.

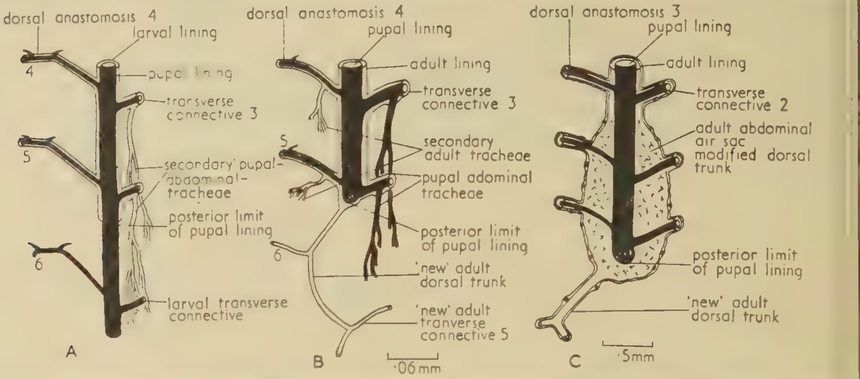

FIG. 11. Tracheae from the mid-abdominal region—right side. A, *Drosophila*: showing relatio of third larval to pupal tracheal linings. B, *Drosophila*: showing relation of pupal to adul tracheal linings, C, *Calliphora*: showing relation of pupal to adult tracheal linings.

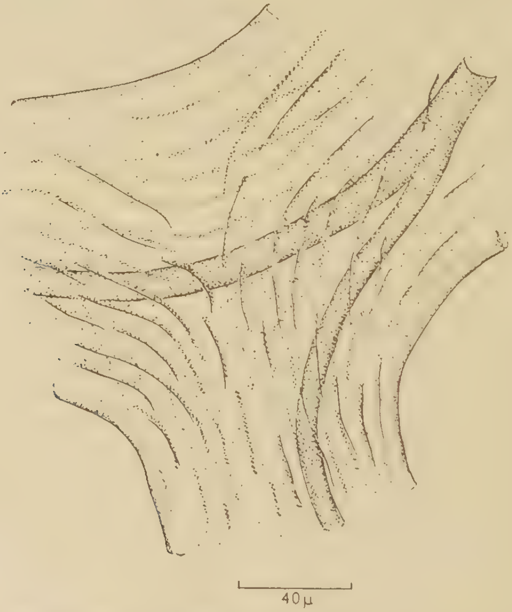

FIG. 12. Portion of lateral trunk and second leg trachea showing pupal tracheal tubes surrounded by folded intima of future adult 'air sac', as seen under oil immersion after double injection with cobalt sulphide.

Certain general principles have, however, already emerged. In the first place there is a definite continuity in the tracheal epithelium during the pupal stage. The adult tracheal system does not arise independently of the pupal, but the adult tracheal lining is secreted around that of the pupal by the same tracheal epithelial layer—though possibly not by the same tracheal epithelial cells. Secondly, the majority of the adult air sacs are produced as new branches of the pre-existing system similar to the new branches formed at each of the preceding stages. Thirdly, the lining of certain pupal tracheae is not replaced by that of the adult, so that moulting, as in the case of the larval/pupal ecdysis, is incomplete. Lastly, the origin of the adult system posterior to the fifth pair of spiracles is completely different from that anterior to it. There are no tracheal metameres in this region in the pupal stage. The pupal trunks and their epithelium are seen to end blindly at the level of the fifth pair of spiracles. However, there are two remarkable features about the newly formed posterior adult system. First, the general pattern formed is identical with that of the preceding larval stage, except for the absence of a tenth tracheal metamere, and secondly, there is no distinction or break between the pattern in the functional anterior and posterior regions, and continuity in the tracheal trunks is maintained. However, from fig. 11, B it can be seen that the longitudinal axis of the dorsal trunk posteriorly is not continuous with the trunk more anteriorly, but comes off instead more medially and at an angle. It is also significantly narrower in diameter, a characteristic remarked on but not explained by Miller (1950). It is clearly due to the fact that posteriorly it is a new development, but anteriorly is the final product of successive secretions of new tracheal tubes outside the preceding tracheal tubes.

DISCUSSION

The nature and origin of the tracheae in the different instars

The form of the tracheal system at the various stages from first larval instar to adult is explained by the developmental processes involved: the peculiarities of each particular stage are due to the combined effect of the method and extent of secretion of the new tracheal linings, the method and extent of the separation and withdrawal of the old linings, together with the general fate of the tracheal epithelial cells.

In the case of the three larval instars, the same tracheal epithelium is responsible for the successive secretion of the tracheal linings at all three stages, and moulting is complete. Thus the larval tracheae are divisible into two categories only: (*a*) those constituting the basic framework, and (*b*) new 'branch' tracheae.

In the case of the pupal system, instead of moulting being complete at the previous ecdysis, the linings of certain of the larval tracheae and tracheoles are retained in the pupa. As a result, the tracheal parts are divisible into three categories: (*a*) the basic framework; (*b*) branch formations; and (*c*) those carried over from the third larval stage. It follows that the number and form of the shed larval tracheal linings, which lie within and are attached to the puparium at the functional and non-functional larval spiracles, can be interpreted from a study of the processes involved in their removal. For instance, a mechanical explanation is provided for the 'hole' in the tracheal linings of th first pair of spiracles. The physiological significance of these holes is discusse by Snodgrass (1924) for *Rhagoletis*, and by Robertson (1936) for *Drosophila*

In the same way, the pupal-adult moult is incomplete and the same thre categories of tracheae exist in the adult as in the pupa: (*a*) those forming th basic pattern, (*b*) branch tracheae, and (*c*) tracheae carried over from the preceding pupal, and in some cases larval stages. However, unlike the pupa tracheae, the form of (*a*) and (*b*) may be either 'tracheate' or expanded an sac-like. The tracheae of the posterior abdominal region fall into a fourt category. These are tracheae of which the epithelium appears for the first time during the pupal stage, replacing, functionally, the larval epithelium whic was histolysed earlier in the pupal stage. This adult tracheal epithelium an the linings are continuous with those of the tracheal metameres of the more anterior region: they may even be classified as an exaggerated example o branch tracheae (category (*b*)).

The fate of the tracheal system during development

Since Robertson's (1936) results on the development of this system are quoted by Bodenstein (1950) in the most recent and comprehensive work on all aspects of *Drosophila* morphology, anatomy, and development, it is important to compare the results given by these workers with those obtained in the present investigation. The following tabulation of events taking place in the tracheal system at 25° C—arrived at from the study of serial sections of accurately timed stages—is selectively quoted from Bodenstein after Robertson. The numbers are inserted by the present author for subsequent comparison.

1. *At 97 hours.* The imaginal lateral spiracles, originating as outgrowths from imaginal cells on the great lateral trunks have connected with the hypoderm.
2. *At 98 hours.* The imaginal spiracles form a tube filled with newly secreted chitin.
3. *At 99 hours.* The larva contracts away from the anterior end of the puparium and partly withdraws the anterior tracheal linings.
4. *At 100 hours.* The pre-pupal moult occurs; a lumen appears in the imaginal abdominal tracheal tubes, which connects the tracheal tracts with a pore in the pupal membrane. The stigmata are probably not functional.
5. *At 108 hours.* Pupation occurs. The anterior and posterior tracheal linings are shed. The newly formed tracheal tube linings of the pre-pupal stage are also withdrawn (*a*). The newly formed system in the head is everted (*b*). The newly formed imaginal pro-thoracic spiracles connect with the lateral larval trunks (*c*).
6. *At 110 hours.* The abdominal tracheae are still larval.
7. *At 114 hours.* The pupal intima of the abdominal longitudinal trunks is broken off near the posterior end.
8. *At 120–156 hours.* The posterior tracheal trunks are histolysed. The

hitinous intima shrinks into the anterior tracheal trunks which form the large horacic air sacs.

9. *At 158 hours.* The closing device of the imaginal stigmata is established.

10. *At 192 hours.* Emergence occurs. The chitinous intima is shed through he pro-thoracic spiracles.

Although the results of the present work indicate the same time sequence, he interpretations of the fate of the tracheal system are quite different from hose outlined above. The above interpretation of the facts available is consistent with the fragmentary evidence and with the generally accepted theory hat the imaginal tracheal system is an entirely new formation directly replacing the larval, part of which is then histolysed and part withdrawn with the cuticle. The results of the present investigation would suggest the following drastic modification of the interpretations of the sequence of events given in Bodenstein (1950).

1. *97 hours.* The so-called 'imaginal spiracles' are in fact the pupal spiracles (see p. 134); they develop mesad to the larval spiracles and communicate with the future pupal tracheal system.

2. *98 hours.* The pupal spiracles do indeed form a tube which is the pupal intima enclosing the fluid-filled lumen. It bears a similar relationship to the larval spiracle as did the third instar spiracle to the second, that is, after ecdysis the opening in the pupal cuticle through which the attached trachea is removed closes and an ecdysial scar remains. Although not conspicuous in *Drosophila*, this can be seen clearly in *Calliphora*.

3. *99 hours.* The tracheal linings are not partly withdrawn at this stage, if 'withdrawn' suggests a partial break in the system and actual removal of the tracheae. The pro-thoracic spiracular tracheae are indeed stretched almost to what would appear to be their maximum degree of taughtness, but origins of the first dorsal anastomosis and ventral and dorsal cervical tracheae and lateral and dorsal trunks are still clearly seen. The dorsal and lateral trunks are partly outside the pupal cuticle and have been drawn through the pupal ecdysial tube, but no break has yet occurred in the larval system of this region.

4. *100 hours.* The statement concerning a 'pore' is clearly confused, as also is the statement that the spiracles are probably not functional, since the term 'functional' here conveys the meaning that they play no part in the elimination of the larval system. In fact, the larval system breaks up into ten pairs of tracheal metameres which are withdrawn through the ten pairs of ecdysial openings associated with ten pairs of pupal spiracles; the cast tracheal linings can be seen lying closely apposed to the inner surface of the so-called pre-pupal cuticle.

5. *108 hours.* (*a*) It is difficult to interpret quite what is involved in the statement concerning the shedding of the anterior and posterior tracheal linings together with the newly formed tracheal tube linings of the pre-pupal stage. The latter are referred to previously to this—in 4—as the imaginal abdominal tracheal tubes. (*b*) The newly formed tracheal system refers to the newly developed adult system of the head. In actual fact, they are the functional

head tracheae of the pupal stage. (*c*) It is not the imaginal pro-thoracic but th pupal thoracic spiracles which connect with the lateral and dorsal pupa trunks, and not with the lateral larval trunks.

6. *110 hours*. The intima of the larval abdominal trunks have already bee removed by way of the abdominal spiracles, but the tracheal epitheliu remains and is subsequently histolysed.

7, 8, and 9. Even without contradictory evidence it would be very peculia if the intima of the abdominal region really shrank into the anterior trachea trunks. It is even more unlikely that this intima should subsequently be she through the first thoracic spiracles. The first thoracic spiracles of which stag is not stated: previously to this, only adult spiracles have been mentione Robertson (1936) also states that the pupal spiracles appear to be retained i the adult.

Post-embryonic development of the system in other Diptera

The discrepancies noted above are of particular importance because i appears that this sequence of events for *Drosophila*—a member of the Dipter Cyclorrhapha, Acalypterae—is true also for *Calliphora*—a member of th Diptera Cyclorrhapha, Calypterae. The sequence in both differs considerabl from that seen in the 'lower' Diptera Nematocera, as illustrated by *Sciar coprophila* (Keister, 1948). In the latter case, the same tracheal epitheliu appears to persist through to the adult stage, secreting the successive trachea linings of the four larval, the pupal, and the adult instars. The presence, i the Diptera Cyclorrhapha, of separate fates for the tracheal epithelium in th anterior and posterior regions marks a distinct step in the evolutionary development within the Diptera.

Moulting of the tracheal and tracheolar linings

The sequence of events taking place in the *anterior* tracheal metameres o *Drosophila* is essentially similar to that occurring throughout the body o *Sciara* (Keister, 1948). In both of these Diptera, moulting is complete at th inter-larval ecdyses and incomplete at the larval-pupal and pupal-adul ecdyses. These Diptera differ from *Rhodnius* (Hemiptera) (Wigglesworth 1954) in which the tracheal linings of the tracheoles are never shed; here, a all ecdyses, the new lining of the trachea is continuous with the old lining o the tracheole and only the old lining of the trachea is shed at ecdysis. Wigglesworth (1954) suggests that a possible distinction between tracheoles an tracheae may be that the intima of the former is never shed, whereas the tracheal linings of the latter are, and that in *Sciara* true tracheoles appear only at the third larval instar. However, if this were the case, such relatively large tracheae in *Drosophila* as those arising from the main cervical tracheae in the pupal head, or the adult wing and haltere tracheae, or the ventral ganglionic tracheae of the pupa and adult would all come under this definition of tracheoles, since they are not shed at the appropriate ecdysis.

The fate of the tracheal epithelium during development

In *Drosophila* and also in *Calliphora*, the larval tracheal epithelium closely surrounds the tracheal lining and is composed of large nuclei occurring at scattered intervals in the enclosing strand of cytoplasm. The cell outlines are not distinct and the epithelium resembles the tracheal epithelium of *Sciara* (Keister, 1948) in appearing to be a syncytium containing large scattered nuclei. The spiral thickenings of the new intima become evident some time before ecdysis of the old lining.

The fate of the individual tracheal epithelial cells is not finally settled. All the evidence presented here suggests that in the head, thorax, and anterior abdominal region the same tracheal epithelium persists into the adult stage. However, in the pupal stage changes certainly seem to take place within the epithelium itself. For instance, at this stage the nuclei of the epithelium are more frequent and smaller than in the larval stages. Also, the surface area of the epithelium is greatly increased—particularly in areas which will secrete the sac-like folded linings of the future adult air sacs—and the epithelium has to separate quite a way from the pupal tracheal lining before secreting this adult lining.

The origin of secondary 'branch' tracheae

It remains to be determined whether the secondary tracheae of earlier instars arise in a way similar to that shown for *Rhodnius prolixus* (Wigglesworth, 1954). In the latter they arise by division of cells of the existing epithelium, followed by migration of these cells outwards as branches; the cells subsequently secrete the tracheal lining of the new 'branch' tracheae. It seems likely from a study of the simultaneous and continuous secretion of the new tracheal lining in the 'branch' tracheae and main tracheae that a similar process operates in *Drosophila*. Also the same process appears to occur in the formation of branch tracheae of the adult stage regardless of whether they are tracheate or sac-like, since both types are frequently secreted by adjacent regions of the tracheal epithelium.

Finally, whereas the larval tracheal epithelium in the posterior abdominal region is undoubtedly histolysed, the method of origin and reorganization of the replacing adult tracheal epithelium remains to be determined. Three possibilities present themselves: first, that these last four tracheal metameres are regenerated from 'imaginal clusters'—if this is so a little of the classical theory (Peréz, 1910) of the origin of the adult system remains; secondly, that there is a backward migration of tracheal epithelial cells from the tracheal epithelium surrounding the blind ends of the pupal dorsal and lateral longitudinal trunks; thirdly, that the adult system of this region arises by a combination of these two processes.

The tracheal system and the pre-pupal stage

The present work on the tracheal system cannot ignore the question of the pre-pupal' instar. It has shown that there is certainly no functional pre-pupal tracheal system interposed between the third instar larva and the pupa However, although no positive evidence has emerged on the nature of th secreted membrane, a certain amount of negative evidence throws doubt o its reputed identity as the cuticle of a distinct 'pre-pupal' instar (Snodgrass 1924), corresponding to the fourth larval instar of Diptera Nematocera.

Invariably, when the operculum of the puparium is removed, the objec within the anterior end of the puparium is either the headless pupa or th evaginated pupal head. In the former case removal of the operculum ofte brings about artificial eversion of the head, accompanied by partial or complete removal of the larval spiracular tracheae, through the ecdysial opening in the new pupal cuticle. There is no suggestion—under microscopic examination—of a membrane corresponding to the so-called pre-pupal cuticle surrounding the pupal head; at no time is there any separate cuticle attached t the operculum; if present this is very closely apposed to the inner layer of th puparium. On the other hand, the membrane lies loose in the abdomina region and can, with care, be isolated. Likewise, dissection of the corresponding stage in *Calliphora* has never revealed a separable cuticle in the head region whereas, contrary to Fraenkel's (1938) observation, the pre-pupal cuticle i evident as a separate membrane as far as the level of the pupal first thoraci spiracles. Removal of the operculum reveals the membrane with a 'torn' edg which suggests that it has been torn from the innermost layer of the operculum, with which it appears to be continuous. When pupae of subsequen stages are removed from the puparium, the 'pre-pupal' cuticle, with the larva tracheae lying on its inner surface, is frequently found lying apposed to th pupal cuticle. In such cases the spiracular threads or the posterior spiracula tracheae have been severed from their connexion with the spiracles on th puparial wall during removal from the puparium.

Simultaneously with the withdrawal of the tracheae of the first pair o tracheal metameres and eversion of the pupal head, the larval mouth armatur and stomodaeum are ejected. Similarly, the larval proctodaeum is remove along with the posterior tracheae of the last pair of tracheal metameres According to Robertson (1936) the 'pre-pupal' cuticle extends into the stomodaeum and proctodaeum and also into the spiracles. However, the whole conception of this membrane as a distinct pre-pupal cuticle is difficult to accept after consideration of the development of the tracheal system, since the larval tracheal system is replaced directly by the pupal system which is secreted around it—except in the posterior abdominal region where no pupa system is found. Also retention of the larval tracheae, larval mouth armature and larval stomodaeum and proctodaeum until the pre-pupal moult, appears to be anomalous, although it may be a mechanical necessity.

There is no definite evidence from any of the Cyclorrhapha of the chemica constitution of this membrane or pre-pupal cuticle (Wolfe, 1954, *a*, *b*, 1955) Clearly the problem posed is whether the membrane is epi-cuticular or endo-cuticular in nature. If epi-cuticular it may be defined as a distinct cuticle and the anomaly with respect to the tracheal system remains. If, however, the cuticle proves to be endo-cuticular, its relation to the tracheal system and to the larval stomodaeum and proctodaeum would be more easily understood. Possibly, owing to the peculiar pupation within the last larval skin, it is mechanically essential for the larval epithelium to separate from the larval cuticle, particularly posteriorly. If the epithelium is still secreting endo-cuticle, as it has been doing continuously throughout the third larval instar (Wolfe, 1955), the separate membrane or 'pre-pupal' cuticle would result. Since the epithelium of the spiracles, stomodaeum, and proctodaeum is continuous with the general hypodermis, it would not be surprising if withdrawal of the epithelium, accompanied by continued secretion of the endo-cuticle, resulted in this separable membrane extending into these invaginations also.

There is an additional reason for questioning the true identity of this 'pre-pupal' cuticle. Snodgrass (1924), Fraenkel (1938), and Robertson (1936) liken the pre-pupal instar to the suppressed fourth larval instar of the Diptera Nematocera. Yet it has been shown here that, in *Drosophila*, moulting of the tracheal system is incomplete at the third larval-pupal moult, although complete at the two inter-larval ecdyses. In *Sciara* (Nematocera) (Keister, 1948) moulting is also incomplete at the last larval-pupal ecdysis, but complete at the three preceding inter-larval ecdyses. In both, the tracheal system of the larval instar prior to the pupal instar shows incomplete moulting. If a suppressed fourth instar—or 'pre-pupa'—intervenes in the Cyclorrhapha, the tracheal system of this instar has disappeared. Also, the distinctive characteristics of the fourth larval-pupal ecdysis in the Nematocera have been taken over by the third larval-pupal ecdysis in the Cyclorrhapha. (Subsequent work on the pre-pupal cuticle forms the basis of a further publication.)

I should like to thank Professor V. B. Wigglesworth for suggesting the problem and for his continual help and encouragement, particularly during the preliminary stages of this work in Cambridge, where the general events taking place in the head and thorax were studied. Acknowledgement is also made of the facilities provided by Queen Mary College for continuing work on the problems presented by the pupal and adult abdomens, and for studying the details of moulting in the various stages.

REFERENCES

Bodenstein, D., 1950. *Biology of Drosophila*, edited by M. Demerec. New York (Wiley).

Chapman, R. N., 1918. *The wings of insects*, by J. H. Comstock. New York (Comstock).

Fraenkel, G., 1938. 'The number of moults in Cyclorrhaphous flies (Diptera).' Proc. roy. ent. Soc. Lond. A, **13**, 158.

Haskins, C. P., and Enzmann, E. V., 1937. 'Studies on the anatomy of the respiratory system of *Drosophila melanogaster*.' J. Morph., **60**, 445.

Keilin, D., 1944. 'Respiratory systems and respiratory adaptations in larvae and pupae of Diptera.' Parasitology, **36**, 1.

Keister, M. L., 1948. 'The morphogenesis of the tracheal system in *Sciara*.' J. Morph., **83**, 373.

Miller, A., 1950. *Biology of Drosophila*, edited by M. Demerec. New York (Wiley).

Palmén, J. A., 1877. *Zur Morphologie des Tracheensystems*. Helsingfors (Frenckell).

Peréz, C., 1910. 'Recherches histologiques sur la metamorphose des Muscides (Calliphora).' Asch. Zool. esp., Ser. 5T4, 274.

Robertson, C. W., 1936. 'The metamorphosis of *Drosophila melanogaster* including a accurately timed account of the principal morphological changes.' J. Morph., **59**, 351.

Ruhle, H., 1932. 'Das larvale Tracheensystem von *Drosophila melanogaster* Meigen un seine Variabilität.' Z. wiss. Zoöl., **141**, 159.

Snodgrass, R. E., 1924. 'Anatomy and metamorphosis of the apple maggot *Rhagoleti pomonella* Walsh.' J. Agr. Research., **28**, 1.

Whitten, J. M., 1953. *Comparative morphology of the tracheal system in Diptera.* Ph.D. Thesis, Cambridge.

—— 1955. 'A comparative morphological study of the tracheal system in larval Diptera. Part I.' Quart. J. micr. Sci., **96**, 257.

Wigglesworth, V. B., 1950. 'A new method for injecting the tracheae and tracheoles of insects.' Ibid., **91**, 217.

—— 1954. 'Growth and regeneration in the tracheal system of an insect, *Rhodnius prolixus* (Hemiptera).' Ibid., **95**, 115.

Wolfe, L. S., 1954*a*. 'The deposition of the third instar larval cuticle of *Calliphora erythrocephala*.' Quart. J. micr. Sci., **95**, 49.

—— 1954*b*. 'Studies of the development of the imaginal cuticle of *Calliphora erythrocephala*.' Ibid., **95**, 67.

—— 1955. 'Further studies of the third instar larval cuticle of *Calliphora erythrocephala*.' Ibid., **96**, 181.

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